

THE IDENTIFICATION AND PURIFICATION OF CALCIUM CHANNEL
PROTEINS FROM *Zea mays*.

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CONTENTS.

Contents.....	i.
Acknowledgements.....	v.
Abbreviations.....	vi.
Abstract.....	viii.

CHAPTER 1.

INTRODUCTION.....	1.
1.1. Calcium as a second messenger.....	1.
1.2. Calcium regulation of physiological processes..	2.
1.3. Plant calmodulin and Ca^{2+} /calmodulin dependent enzymes in plants.....	4.
1.4. The suitability of calcium.....	5.
1.5. Regulation of cytosolic calcium.....	7.
1.6. Ca^{2+} entry into the cytoplasm.....	9.
1.7. Calcium channels.....	11.
1.8. Methods of studying calcium channels.....	12.
1.9. Mechanism of ion permeation.....	13.
1.10. Selectivity of the calcium channel.....	15.
1.11. Calcium channel antagonists.....	16.
1.12. Different types of calcium channels.....	21.
1.13. The structure of voltage-dependent calcium channels.....	22.
1.14. Regulation of voltage-dependent calcium channels.....	27.
1.15. Calcium channels in plants.....	28.
1.16. Summary.....	31.

CHAPTER 2.

MATERIALS AND METHODS.....	33.
2.1. Plant material.....	33.
2.2. Radiochemicals.....	33.
2.3. Chemicals.....	33.
2.4. Centrifuges.....	34.
2.5. Liquid scintillation counting.....	34.
2.6. pH meter.....	34.
2.7. Preparation of plant tissue.....	35.
2.8. Preparation of crude membrane fractions.....	35.
2.9. Solubilisation of the crude membrane fractions.	36.
2.10. Phase partitioning of crude membrane.....	36.
2.11. Verapamil binding assays.....	37.
2.11.1. Centrifugation method.....	37.
2.11.2. Filter method.....	38.
2.12. [^3H] LU 49888 binding assay.....	39.
2.13. Column chromatography.....	40.
2.14. DEAE ion exchange chromatography.....	42.
2.14.1. Linear NaCl elution gradient.....	42.
2.14.2. Step NaCl elution gradient.....	42.
2.14.3. Final DEAE elution protocol.....	43.
2.15. Gel filtration column chromatography.....	43.
2.15.1. Sephacryl S300 column.....	43.

2.15.2. Sephacryl S400 column.....	44.
2.16. Hydrophobic interaction chromatography.....	45.
2.16.1. Phenyl Sepharose column buffers.....	45.
2.16.2. Basic elution protocol.....	45.
2.16.3. Final phenyl Sepharose elution protocol.....	46.
2.17. Lectin Affinity chromatography.....	48.
2.17.1. Con-A Sepharose elution protocol.....	48.
2.17.2. Wheat germ agarose elution protocol.....	49.
2.18. SDS Polyacrylamide Gel Electrophoresis.....	49.
2.18.1. Equipment.....	49.
2.18.2. Gel preparation.....	50.
2.18.3. Sample preparation.....	50.
2.18.4. Running conditions.....	51.
2.18.5. Molecular weight markers.....	51.
2.19. Visualisation of proteins in polyacrylamide gels.....	52.
2.19.1. Coomassie blue staining.....	52.
2.19.2. Silver staining.....	52.
2.20. Photography.....	53.
2.21. Protein determination.....	54.
2.22. Protein Western Blotting.....	54.
2.23. Visualisation of proteins blotted onto nitrocellulose.....	55.
2.24. Binding of [³ H] verapamil to proteins immobilised onto nitrocellulose.....	55.
2.24.1. Method 1.....	56.
2.24.2. Method 2.....	56.
2.24.3. Method 3.....	57.
2.25. Binding of Con-A to proteins immobilised onto nitrocellulose.....	58.
2.26. Binding of ⁴⁵ Ca ²⁺ to proteins immobilised onto nitrocellulose.....	58.
2.27. Photoaffinity labelling of the phenylalkylamine binding protein.....	59.
2.27.1. Method 1.....	59.
2.27.2. Method 2.....	60.
2.28. Protein kinase assays.....	61.
2.28.1. Buffers.....	61.
2.28.2. Cellulose disc assay.....	62.
2.28.3. Phosphocellulose strip assay.....	62.

CHAPTER 3.

THE CHARACTERISATION OF [³ H] VERAPAMIL BINDING IN CRUDE MEMBRANE PREPARATIONS FROM MAIZE COLEOPTILES...	64.
3.1. Introduction.....	64.
3.2. Principles of the binding assay.....	65.
3.3. Development of the [³ H] verapamil binding assay.....	66.
3.3.1. The centrifugation assay.....	66.
3.3.2. The filter assay.....	68.
3.4. The kinetics of [³ H] verapamil binding to maize coleoptile membranes.....	69.

3.5.	[³ H] verapamil and [³ H] D888 binding to maize coleoptile membranes.....	69.
3.6.	Specific binding constants of verapamil and D888 and the estimation of binding protein density.....	74.
3.7.	The displacement of [³ H] verapamil and [³ H] D888 by unlabelled calcium channel antagonists.	77.
3.7.1.	[³ H] verapamil displacement.....	77.
3.7.2.	[³ H] D888 displacement.....	77.
3.8.	Localisation of [³ H] verapamil binding sites according to tissue type and membrane type.....	80.
3.8.1.	Tissue type.....	80.
3.8.2.	Membrane type.....	84.
3.9.	Discussion.....	86.

CHAPTER 4.

THE PARTIAL PURIFICATION OF A VERAPAMIL BINDING PROTEIN FROM MAIZE COLEOPTILES.

4.1.	Introduction.....	92.
4.2.	Solubilisation of the membrane bound verapamil binding protein.....	94.
4.2.1.	Optimisation of solubilisation.....	94.
4.2.2.	[³ H] verapamil binding in CHAPS solubilised membrane fractions.....	102.
4.3.	DEAE ion exchange column chromatography.....	105.
4.3.1.	Development of the elution protocol.....	106.
4.3.2.	The final elution protocol.....	109.
4.4.	Gel filtration column chromatography.....	111.
4.4.1.	Sephacryl S300 experiments.....	111.
4.4.2.	Sephacryl S400 experimemnts.....	114.
4.5.	Hydrophobic Interaction column chromatography..	116.
4.5.1.	Development of the elution protocol.....	117.
4.5.2.	The final elution protocol.....	127.
4.6.	Lectin affinity column chromatography.....	129.
4.7.	SDS Polyacrylamide gel electrophoresis.....	134.
4.8.	The purification procedure: Summary and discussion.....	138.

CHAPTER 5.

IDENTIFICATION AND VISUALISATION OF THE VERAPAMIL BINDING PROTEIN FROM MAIZE COLEOPTILES.....

5.1.	Introduction.....	148.
5.2.	Photoaffinity labelling using [N-methyl- ³ H] LU49888.....	148.
5.2.1.	[N-methyl- ³ H] LU 49888 binding in crude membrane preparations from maize coleoptiles.....	148.
5.2.2.	Photoaffinity labelling of the phenylalkylamine binding protein(s) from maize coleoptile	

<i>membrane preparations</i>	149.
5.3. [³ H] verapamil binding to Western blots of crude membrane fractions and to fractions containing the partially purified putative verapamil binding protein.....	156.
5.4. Discussion.....	158.

CHAPTER 6.

THE PRELIMINARY CHARACTERISATION OF THE PUTATIVE VERAPAMIL BINDING PROTEIN.....	160.
6.1. Introduction.....	160.
6.2. The possible glycoprotein nature of the putative verapamil binding protein.....	161.
6.3. Is the putative verapamil binding protein a calcium binding protein?.....	162
6.4. The possible control of plant calcium channels by phosphorylation.....	164.
6.5. Discussion.....	169.

CHAPTER 7.

DISCUSSION.....	173.
7.1. The experimental approach: Problems and criticisms.	
7.1.1. Using [³ H] verapamil as a marker for calcium channels.....	173.
7.1.2. Purification of the verapamil binding protein.....	175.
7.2. The need for calcium channels in plants.....	175.
7.3. What are the functions of plant calcium channels?.....	177.
7.4. Properties of plant calcium channels.....	179.
7.5. Future work.....	181.

REFERENCES.....	184.
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APPENDIX.....	197.
Publication.....	198.

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ABBREVIATIONS.

A ₂₈₀	absorbance at 280 nm.
A ₅₉₅	absorbance at 595 nm.
AMPS	ammonium persulphate.
cAMP	cyclic adenosine monophosphate.
[γ ³² P] ATP	[γ ³² P] labelled adenosine triphosphate.
B _{max}	maximum number of binding sites.
β PBD	2-(4'-tetra-butylphenyl)-5-(4''-biphenyl)-1,3,4-oxadiazole.
BSA	bovine serum albumin.
CAM	calmodulin.
CHAPS	[3-(3-cholamidopropyl) dimethylammonio] propane-1-sulphonate.
Ci	curie.
Con-A	Concanavalin A.
CPM	counts per Minute.
D600	methoxyverapamil; (2,7-dimethyl-3-(3,4,5-trimethoxyphenyl)-3-cyan-7-aza-9-(3,4-dimethoxyphenyl)-nonanhydrochloride.
D888	desmethoxyverapamil; (2,7,-dimethyl-3-(3,4,5-trimethoxyphenyl)-3-cyan-7-aza-9-(3-methoxyphenyl)-nonanhydrochloride.
Da	dalton.
DEAE	diethylaminoethyl.
DHP	dihydropyridine.
d.H ₂ O	distilled water.
d.d.H ₂ O	deionised distilled water.
DTT	dithiothreitol.
EDTA	ethylenediamine tetra-acetic acid..
EGTA	ethylene glycol bis-(β -aminoethyl ether)N,N,N',N'-tetra-acetic acid.
ER	endoplasmic reticulum.
GF/C	glass fibre, grade C.
HEPES	N-2-Hydroxyethyl piperazine-N-2-ethanesulfonic acid.
HRP	horse radish peroxidase.
IC ₅₀	inhibition constant; concentration at which 50% of the specifically bound radioligand is displaced.
K _D	Dissociation constant.
kDa	Kilodalton.
LU 49888	[N-methyl- ³ H] Azidopamil; (-)-5-[(3-azidophenethyl) [N-methyl- ³ H] methylamino]-2-(3,4,5-trimethoxyphenyl)-2-isopropyl-valeronitrile.
mA	milli Ampere(s).
Mops	(3-[N-Morpholino] propanesulfonic acid).
Mr	molecular ratio.
mS	milli Second.
NaPP	Sodium pyrophosphate.
Nifedipine.	1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester.

NTC	nitrocellulose.
PAGE	polyacrylamide gel electrophoresis.
PEG	polyethyleneglycol.
PEI	polyethyleneimine.
Pi	inorganic phosphate.
SDS	sodium dodecyl sulphate.
TBS	tris buffered saline.
TCA	trichloroacetic acid.
TEMED	N,N,N',N'-tetramethylethylene-diamine.
Tris	tris (hydroxymethyl)-aminoethane.
Triton X-100	iso-octylphenoxy polyethoxyethanol.
UV	ultraviolet.
Verapamil	(2,7,-dimethyl-3-(3,4-dimethoxyphenyl)-3-cyan-7-aza-9-(3,4,-dimethoxyphenyl)-nonanhydrochloride.
v/v	volume for volume.
WGA	wheat germ agarose.
w/v	weight for volume.
w/w	weight for weight.

ABSTRACT.

Binding of the calcium channel antagonist [^3H] verapamil has been detected in crude and solubilised membrane fractions from maize coleoptiles. Binding was saturable and reversible. The dissociation constants for specific binding of [^3H] verapamil to crude and solubilised membrane fractions were 72 nM and 158 nM respectively, with respective binding-site concentrations of 135 pmol/mg protein and 78 pmol/mg protein. In both cases the Scatchard plots were linear indicating a single class of non-interacting binding sites.

[^3H] verapamil binding to crude maize coleoptile membrane fractions could not be displaced by unlabelled desmethoxyverapamil or nifedipine, but could be displaced by methoxyverapamil. These results suggest that there may be alternative specific binding sites for the different channel antagonists in plant membranes, possibly within the same channel. Preliminary investigations suggest that the number of calcium channels varies according to tissue type and that there is subcellular localisation of verapamil binding calcium channels on the plasma membrane.

Partial purification of a verapamil binding protein was achieved using detergent solubilisation of the membrane bound protein(s), followed by ion exchange, gel filtration and hydrophobic interaction chromatography. This resulted in an average purification of 36 ± 16 fold

(n=7). Analysis of the polypeptides from the final purified fraction, by SDS polyacrylamide gel electrophoresis, indicated that a protein of 169 kDa may contain the verapamil binding site. However the verapamil binding protein could not be further accurately identified either by photoaffinity labelling or by [³H] verapamil overlay techniques. Preliminary experiments also suggested that the putative verapamil binding protein and possible associated channel proteins, were not glycosylated, did not bind calcium and were not phosphorylated by endogenous protein kinase.

CHAPTER 1.

INTRODUCTION.

The aim of this introduction is to describe what is known about the role of calcium in the regulation of plant physiology and how cytoplasmic calcium levels are maintained in plant cells. This will be followed by an outline of our knowledge to date about calcium channels in both plants and animals and their importance in the regulation of intracellular calcium levels.

1.1. Calcium as a second messenger.

Animal and plant cells need to convert extracellular signals into an intracellular message that can be understood by the cells to achieve the correct biochemical or physiological response. In animal cells, calcium ions, together with cyclic AMP, act as second messengers to translate primary signals, such as light and hormones, into a final response. Cyclic AMP, although present in higher plant cells (Brown & Newton, 1981), does not appear to have a role as a second messenger; the only known primary receptor for cyclic AMP, a cAMP-dependent protein kinase has never been found and cAMP has not been shown to be required for any physiological response (Saliamath & Marmé, 1983). Calcium ions alone, therefore, may be the main second messenger in plant cells, coupling stimulus to response and co-ordinating plant processes. There is also some evidence to suggest

that, as in animal systems, there is interaction between calcium and the inositol phospholipids to create an intracellular messenger system (Elliot & Skinner, 1986; Reddy *et al.*, 1987; Ranjeva *et al.*, 1988).

The following sections will focus on calcium as an intracellular regulator of plant processes and how the plant cell can achieve this regulation.

1.2. Calcium regulation of physiological processes in plants.

Calcium has long been known to be an important macronutrient in the growth and development of plants (Kirkby & Pilbeam, 1984). Recently it has become increasingly apparent that calcium ions are also a major intracellular regulator of numerous biochemical and physiological processes. Such processes include polarised growth (Nobling & Reiss, 1987; Brownlee & Wood, 1986; Hepler & Wayne, 1985), cytoplasmic streaming (Hayama *et al.*, 1979; Williamson & Ashley, 1982) and mitosis (Keith *et al.*, 1985; Hepler & Callaham, 1987).

The involvement of calcium has been shown by altering cytoplasmic calcium with either calcium ionophores, by application of calcium antagonists or by chelation of extracellular calcium. A number of workers have also demonstrated that calcium ions mediate a variety of events elicited by extracellular signals, including light, hormones and gravity (Wayne & Hepler, 1985; Roux *et al.*, 1986; Elliot, 1986; DeSilva *et al.*, 1985;

Poovaiah *et al.*, 1987). A change in cytosolic calcium concentration caused by the extracellular signal is considered to be the primary event in triggering a cellular response. It is proposed that the signal causes a rise in cytosolic calcium concentration which then elicits the response.

One of the difficulties involved in determining the role of calcium in signal-response coupling, is the measurement of cytoplasmic calcium levels. There are various methods available for such measurement, for example, calcium-binding dyes, calcium-activated photoproteins and calcium-sensitive electrodes. However, technical problems arise when applying them to plants. Despite this, some measurements have been made, which set cytosolic calcium concentration at approximately 10^{-7} M in plant cells (Gilroy *et al.*, 1986; Williamson & Ashley, 1982; Brownlee & Wood, 1986). The change in internal calcium concentration in response to an extracellular signal e.g. light, has also been demonstrated (Miller & Sanders, 1987).

Calcium then, appears to regulate a number of physiological processes in plants. The next sections show that plant cells have the means to sense and respond to external stimuli and so elicit these processes and that calcium as an ion is ideally suited to this role.

1.3. Plant calmodulin and Ca^{2+} -calmodulin dependent enzymes in plants.

Calmodulin consists of a single polypeptide chain of 148 amino acids with four calcium binding domains which bind calcium with high affinity. When cytosolic calcium concentration rises, calmodulin becomes functionally active and binds calcium. It then undergoes a conformational change which allows it to interact with target proteins and modulate their functions (Klee & Vanaman, 1982).

Calmodulin has been found in plant tissues (Anderson *et al.*, 1980) and is very similar to animal calmodulin. The amino acid sequence of spinach calmodulin has only 13 different amino acids to that of bovine brain calmodulin. This difference does not appear to cause any functional changes and the affinities of plant and vertebrate calmodulin for calcium appear to be of the same order of magnitude (Marmé *et al.*, 1984).

The Ca^{2+} -calmodulin complex, once formed, plays an important role in the messenger system by directly regulating certain enzymes or by activating a protein kinase for example, which can regulate other enzymes/proteins by phosphorylation. These two modes of action allow fast and slow responses to be mediated by calcium and calmodulin. The first calmodulin-dependent enzyme to be discovered in plants was NAD kinase from peas (Anderson & Cormier, 1979). Other enzymes have since been shown to be Ca^{2+} -calmodulin dependent. These include

other NAD kinases localised in the cytoplasm, mitochondrial membranes and chloroplast envelope (Dieter & Marmé, 1980), Ca^{2+} and Mg^{2+} ATPases (Fukumoto & Venis, 1986), protein kinases (Blowers *et al.*, 1985) and Quinate NAD^+ oxidoreductase (Graziana *et al.*, 1983).

The existence of calmodulin and Ca^{2+} -calmodulin dependent enzymes in plant cells suggests that plants have the biochemistry to use calcium as a second messenger. Fig.1.1. illustrates a possible scheme for calcium as a second messenger in plants.

1.4.The suitability of calcium.

Calcium itself has various properties which make it a suitable ion to regulate intracellular processes, compared to other ions such as magnesium, sodium or potassium.

At elevated levels (approaching millimolar), calcium will react with inorganic phosphate forming an insoluble precipitate which inhibits the normal phosphate based energy metabolism of the cell. Therefore it is important that the cell maintains a low ($0.1 \mu\text{M}$ - $1.0 \mu\text{M}$) intracellular concentration of calcium.

By having a low concentration of cytosolic calcium, a small change in the absolute amount of calcium in the cytosol can result in a 10 - 100 fold rise ($0.1 \mu\text{M}$ - $1.0 \mu\text{M}/10 \mu\text{M}$) which can trigger a physiological response without greatly disturbing the ionic environment of the

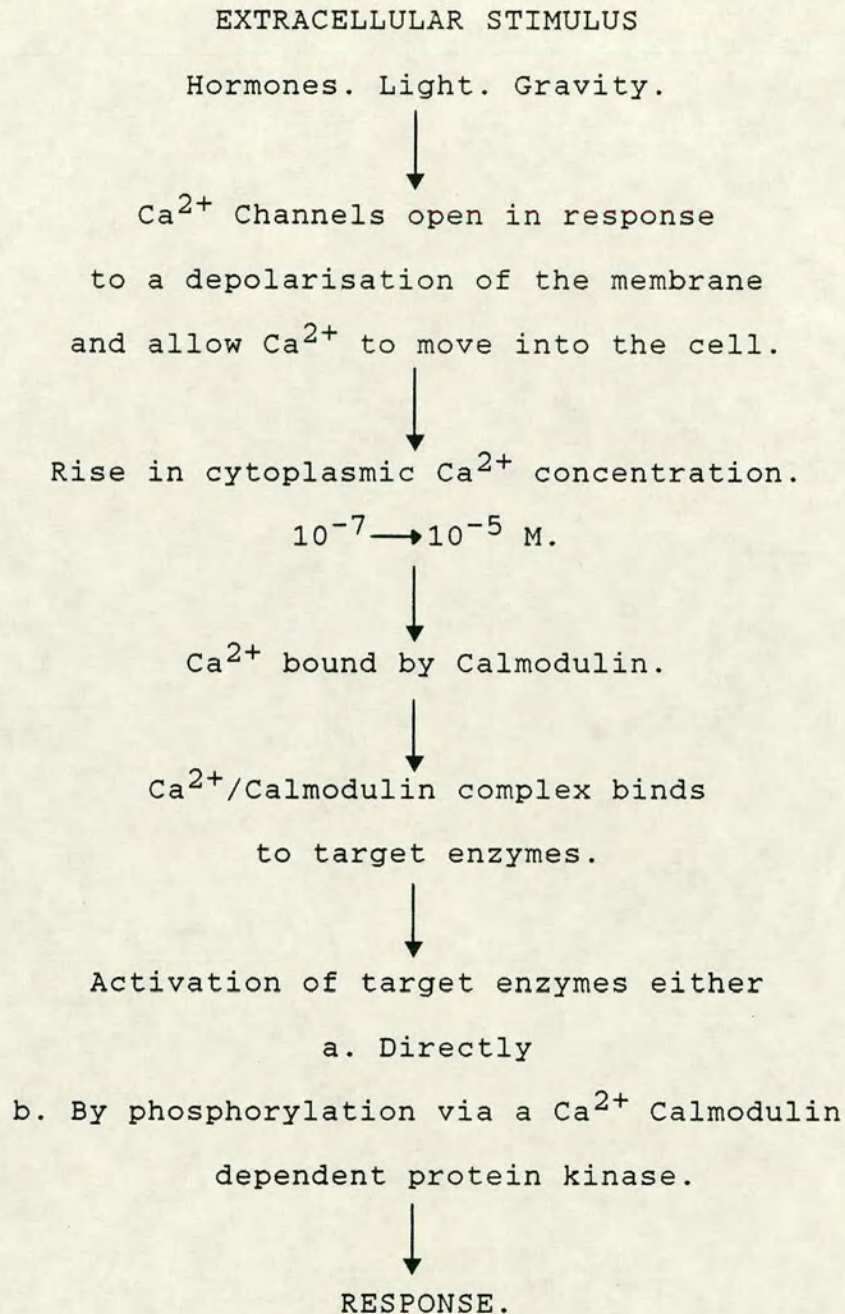


Fig.1.1. The possible role of calcium as a second messenger in plant cells.

cell. The influx of a relatively small number of ions is not an osmotic burden to the cell, as they can be pumped out, bound or sequestered, to return the cell to its resting calcium levels with very little energy expenditure. The rapid removal of ions allows both spatial and temporal calcium gradients to form which can carry a message from the initial signal. In the case of the more abundant ions like Mg^{2+} or K^+ , the cell would have to increase the concentration of these ions from 10^{-3} - $10^{-1}M$ to achieve the same effect. This would upset the osmotic balance of the cell and require a lot of energy to restore the cell to its resting level.

Calcium also has a low free energy of hydration which allows a more rapid exchange of water molecules, (approximately 10^4 times faster than Mg^{2+}), when binding to target sites. Interaction with a receptor is therefore faster and requires less energy. The variable coordination number and bond length of the calcium ion also allows greater flexibility of interaction with receptor sites compared to ions like Mg^{2+} which have fixed coordination and bond lengths.

1.5. Regulation of cytosolic calcium.

Due to the low internal concentration of calcium there is a large difference, (1000-10,000 fold), in calcium concentration between the cytoplasm and cell exterior. This results in a large potential driving force for inward calcium movement. However, the permeability of

the membrane for calcium ions is low and so calcium movement into the cell is slow. Only when the calcium channels are open does the calcium move rapidly down its electrochemical gradient into the cell, so causing a rise in cytosolic calcium levels. To return the cytosolic calcium concentration to its low level requires that the ion be actively pumped out of the cytosol to the apoplast or sequestered into other cell organelles including vacuoles, endoplasmic reticulum, chloroplasts and mitochondria (Evans, 1988). A number of calcium pumps that can do this have been identified in the various membrane fractions including plasma membrane (Dieter & Marmé, 1981), endoplasmic reticulum (Schumaker & Sze, 1986) and mitochondria (Hodges & Hanson, 1965; Dieter & Marmé, 1980). Chloroplasts have also been shown to sequester calcium when illuminated (Kreimer *et al.*, 1985; Miller & Sanders, 1987). The plasma membrane calcium pump is probably the major pump regulator of cytosolic calcium, although in daylight, chloroplasts in photosynthesising tissue may play an important regulatory role. The calcium pumps themselves appear to be regulated by a number of factors including plant growth substances (Kubowicz *et al.*, 1982), phytochrome (Roux *et al.*, 1986), light (Miller & Sanders, 1987) and calmodulin (Dieter & Marmé, 1983; Moore & Akerman, 1984; Fukumoto & Venis, 1986). Calmodulin probably has a homeostatic effect by stimulating the plasma membrane pump as the cytosolic calcium rises, resulting in the restoration of lower

calcium concentrations in the cytoplasm. Calmodulin also plays another role in maintaining a low internal calcium concentration by binding calcium ions when they enter the cell. These functions, although important to regulation of cytosolic calcium, are minor compared to the function of calmodulin in processing the extracellular signal.

An outline of intracellular calcium regulation is summarised in Fig. 1.2.

1.6. Calcium entry into the cytoplasm.

This introduction has so far considered the mechanisms involved in the maintenance of low cytosolic calcium concentration and the role of calcium as a second messenger in plants. Central to this role of intracellular messenger is the rise in cytoplasmic calcium triggered by the primary stimulus. In animal cells the stimulus causes the release of calcium from intracellular organelles and/or causes plasma membrane sited calcium channels to open, through which calcium ions move into the cell.

There are two main types of calcium channels; the voltage-dependent calcium channel and the receptor-operated channel, (Benham & Tsien, 19867; Neher, 1987). The receptor-operated channels, which appear to be independent of voltage and are linked to phospholipid metabolism, appear to be the type responsible for calcium release from intracellular organelles such as endoplasmic reticulum in animal cells (Michell, 1982; Berridge,

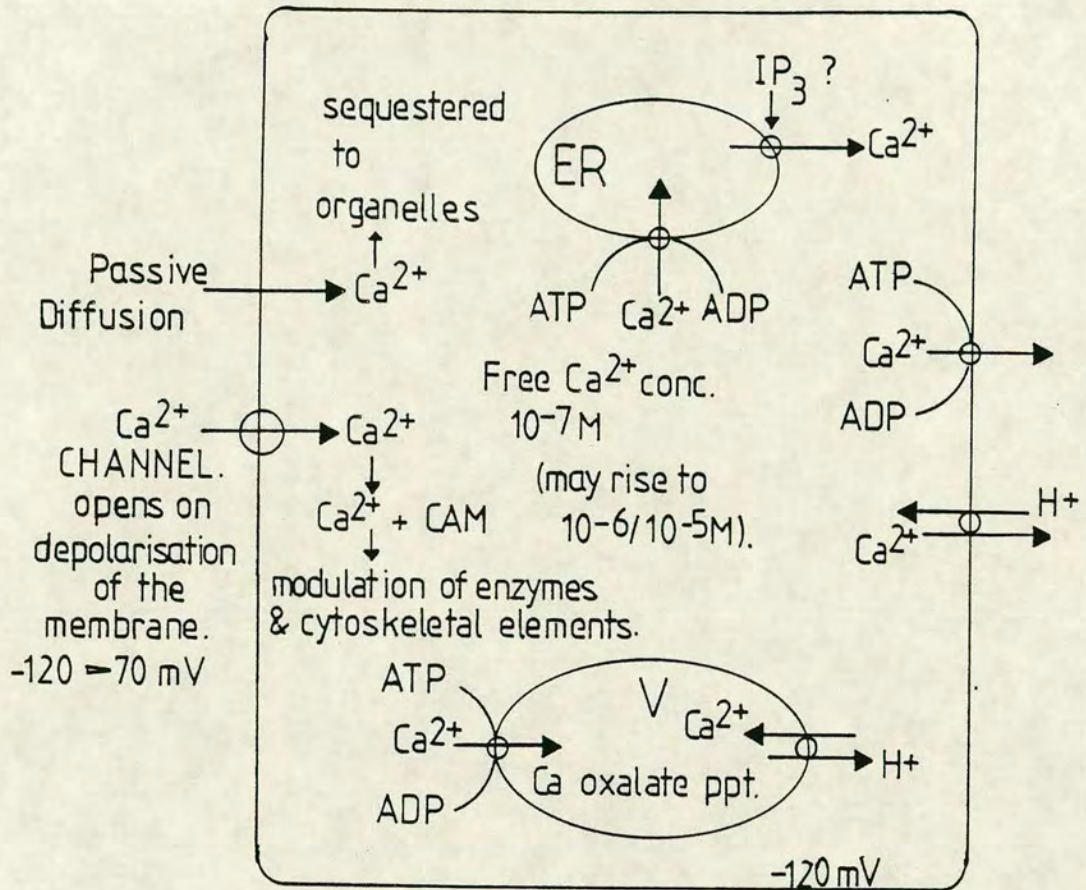


Fig.1.2. A pictorial summary of intracellular calcium regulation in plant cells.

The inter-relationships between the various mechanisms used by a plant cell to regulate intracellular calcium levels are shown. V denotes the vacuole. ER denotes the endoplasmic reticulum.

1986). It is possible that receptor operated channels may exist in plants to allow release of calcium from organelles, but there is no firm evidence for them, as indeed there is no firm evidence for the involvement of phospholipid metabolism in the second messenger system in plants.

Calcium entry through the plasma membrane sited voltage-dependent calcium channels, is probably the most important route whereby cytosolic calcium is increased. In the remaining sections of this introduction some information about voltage-dependent calcium channels in animal cells will be presented, followed by what is known about calcium channels in plant cells.

1.7. Calcium channels.

Calcium channels are membrane proteins which form pores that are capable of transferring millions of calcium ions per second from the cell exterior into the cytoplasm (Tsien, 1983). The channels open in response to a change in membrane potential (usually a depolarisation), and allow calcium ions to move down their electrochemical gradient into the cytoplasm (Hagiwara & Byerly, 1983; Tsien, 1983; Reuter, 1983). The calcium flux creates a net inward, depolarising current and results in the accumulation of calcium in the cytoplasm. In the case of animal cells, the rise in cytosolic calcium effects such processes as muscle contraction, secretion of hormones and neurotransmitters,

the regulation of enzyme activities and other calcium sensitive events (Reuter, 1983). The calcium channels play a crucial role in coupling membrane excitation to cellular responses and are a major way of controlling internal calcium.

1.8. Methods of studying calcium channels.

Calcium channels have been studied using a combination of electrophysiological, pharmacological and biochemical techniques. Using the electrophysiological technique of patch clamping, a very small area of membrane can be electrically isolated from its surroundings. The patch of membrane may contain one or more functional calcium channels, which can open and close as the membrane potential is changed. The current flow across the membrane patch (i.e. through the channels) into the pipette can be collected and recorded. From this channel activity can be identified. Patch clamping can be applied to whole cells or to single channels (Hammil *et al.*, 1981; Lee *et al.*, 1980).

Patch clamp recordings have allowed the study of the mechanism of ion permeation and selectivity of calcium channels (Hess & Tsien, 1984), the identification and characterisation of multiple channel types in various tissues eg; sensory neurons and ventricular heart cells (Reuter, 1986; M^CClesky *et al.*, 1986) and the mechanisms of calcium channel modulation by hormones, neurotransmitters and drugs which alter calcium entry

into cells by increasing or decreasing channel activity (Lee & Tsien, 1983; Reuter, 1983).

Pharmacology has also been employed to study calcium channels. Different calcium channel types have different sensitivities to various pharmacological agents and can be distinguished between on that basis (Reuter, 1986). Such agents include various neurotransmitters, hormones, toxins and a group of drugs known as calcium antagonists and agonists. As well as modulating calcium entry, these drugs have been used biochemically in the purification of voltage dependent calcium channels and to elucidate their structure (Glossmann & Streissnig, 1988).

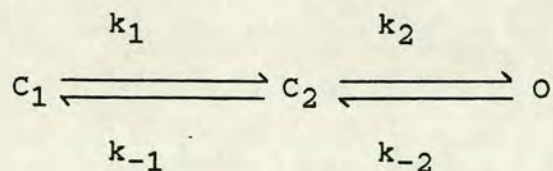
1.9. Mechanism of ion permeation.

In excitable membranes of animal cells, calcium channels are controlled by voltage-dependent gating, i.e. the opening and closing kinetics are a result of changes in membrane potential. Evidence suggests that a 'voltage sensor' within the membrane, possibly a protein with dipole properties, that is an integral part of the channel, reacts to the electric field. Any change in membrane potential will cause a reorientation of the charged 'sensor' within the field and thus a change in the ion flow through the channel (Reuter, 1983).

The probability that a channel will be open depends on membrane potential, as does the current that flows while the channel is open. Studies employing patch clamping of single calcium channels from cardiac cells

show that as depolarisation occurs the probability of the channel being open increases. A steady level of activation is usually reached in a few milliseconds (Reuter *et al.*, 1982). During long depolarising steps calcium channels can show inactivation i.e. a decline in calcium conductance through the channel. This is 20-30 times slower than activation and maybe caused by membrane potential (which may decrease the probability of the channel being available to open) or increased intracellular calcium. Some excitable cells show little inactivation, while others display voltage and/or calcium dependent inactivation to different degrees (Tsien, 1983; Reuter *et al.*, 1982). Calcium channel types can be distinguished between on the basis of activation and inactivation properties (see below).

The observed distribution of channel open and closed times (from patch clamp recordings of single channels), have been interpreted in terms of 2 closed states (C_1, C_2) and 1 open state (O), with adjustable rate constants (Fenwick *et al.*, 1982; Tsien, 1983). The following equation describes these states.



Hess *et al.* (1984) have postulated that there are multiple modes of channel gating and that the channel switches between them. Each mode has characteristic open and closing kinetics, shown in the above equation. Mode 1

is characterised by brief openings (1ms) occurring in rapid bursts and probability of openness increases at stronger depolarisations. Mode 2 can be distinguished by relatively long lasting channel openings (20ms) and typically brief closings with the probability of openness being high. The rate constants between $C_1 - C_2 - O$ for mode 1 and 2 are very different. Another mode, mode 0 refers to a condition where the channel is unavailable for opening; no recording of channel opening can be made. Voltage-dependent inactivation is thought to increase the proportion of time a channel spends in mode 0. The transitions between the different modes is thought to be slower than the open/close reactions within the mode, but the exact intermode connections e.g. are they all voltage-dependent or do proteins require covalent modification/phosphorylation before transition can occur (Reuter, 1987), are not known. It is likely that channel gating mechanisms are highly complex, more so than indicated here.

1.10. Selectivity of the calcium channel.

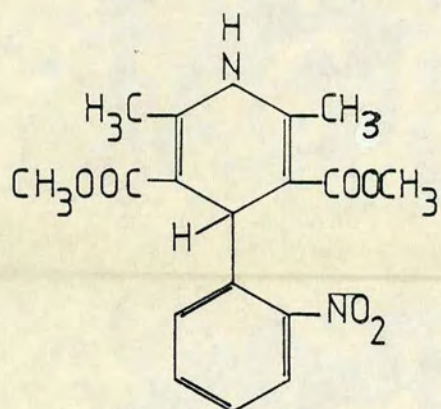
The calcium channel is highly selective for calcium ions despite the fact that in the medium outside the channel, calcium ions are greatly outnumbered by other ions. It has been postulated that the selectivity of the calcium channel is due to the presence of ion binding sites which preferentially bind calcium over any other ions. The channel is therefore occupied almost

continually by one or more calcium ions which, by electrostatic repulsion, guard the channel against the permeation by other ions. The repulsion between calcium ions also allows high throughput rates (Hess & Tsien, 1984; McClesky & Almers, 1985). Recently the binding of the permeant ion to its binding site in the channel and the subsequent change in conformation of the protein, has been shown to effect ion permeation through calcium channels (Pietrobon *et al.*, 1988).

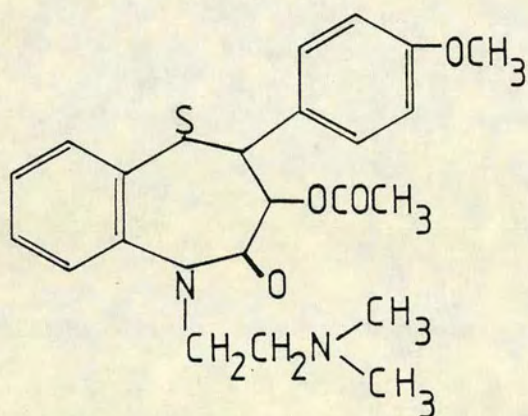
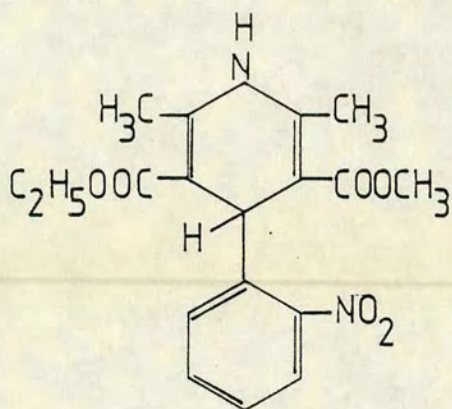
1.11. Calcium channel antagonists.

Calcium channel antagonists and agonists specifically interfere with calcium entry through open channels. Using a structural classification, four main groups of these drugs can be identified. These are the phenylalkylamines e.g. verapamil, desmethoxyverapamil (D888), methoxyverapamil (D600); the dihydropyridines e.g. nifedipine, nitrendipine; the benzothiazepines e.g. diltiazem and the diphenylpiperazines e.g. cinnarizine and flunarizine. Other antagonists include bepridil, fendiline and papaverine. Verapamil, nifedipine and diltiazem are used clinically to treat cardiovascular disorders including angina and hypertension (Triggle *et al.*, 1986). Fig. 1.3. shows the structures of some of these compounds. Inorganic cations such as Mn^{2+} , Cd^{2+} and La^{3+} are non-specific antagonists and also interfere with the conductance of calcium.

NIFEDIPINE

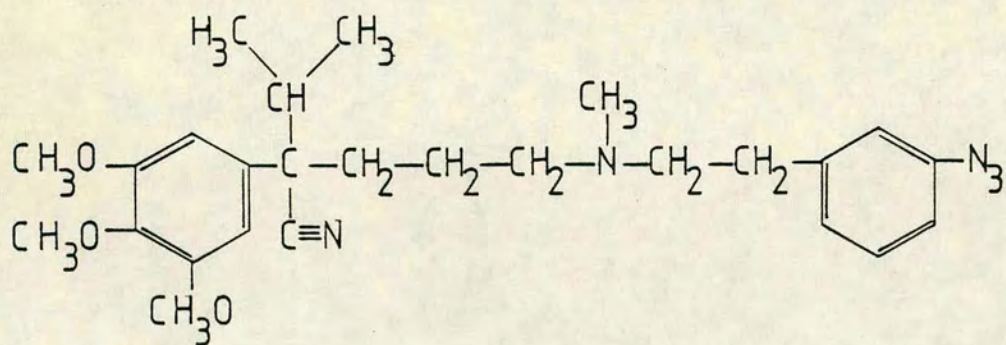


NITRENDIPINE



DILTIAZEM

AZIDOPAMIL LU 49888



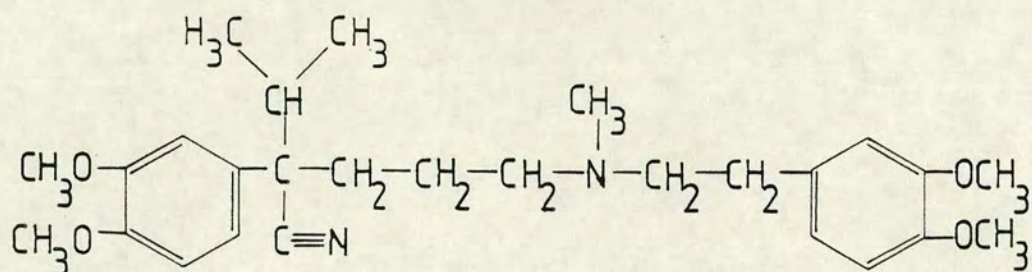
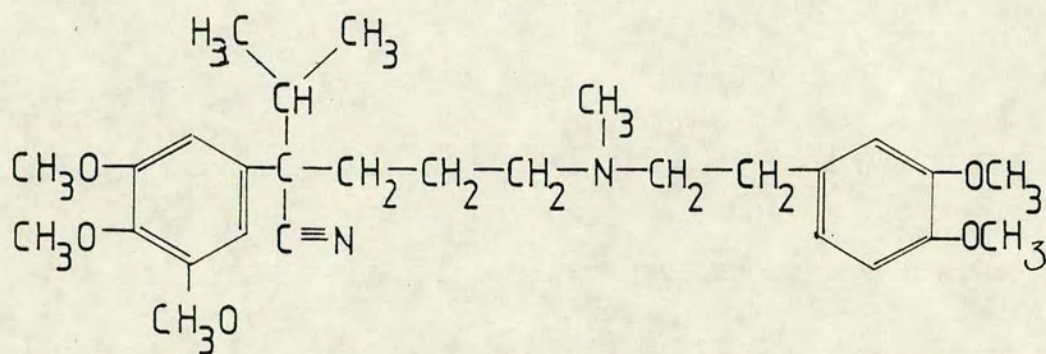
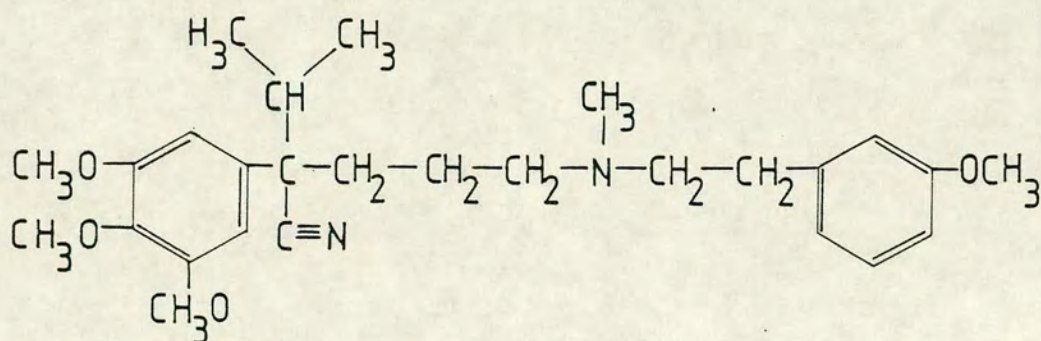
VERAPAMILMETHOXYVERAPAMIL D600DESMETHOXYVERAPAMIL D888

Fig.1.3. The structures of various calcium channel antagonists.

Calcium antagonists exert their primary action on the voltage-dependent channel. Contractions in smooth muscle, (caused by depolarisation of the membrane which opens the calcium channels), are almost completely abolished in the presence of high concentrations of antagonists. $^{45}\text{Ca}^{2+}$ influx induced by depolarisation is also inhibited (Godfraind & Miller, 1986). On repetitive depolarisation the binding site for the compounds becomes more accessible or there is an increase in affinity of the site for the drugs with changes in membrane polarity. This leads to an increase in inhibition of contraction over time which is termed 'use-dependence', a general property of most calcium channel antagonists. This is more pronounced for D600 type drugs than for dihydropyridine antagonists. Diltiazem is intermediate between the two (Lee & Tsien, 1983).

Inhibition of calcium flux may not happen in all situations as calcium channels are heterogeneous (Hagiwara & Byerly, 1983), and may not always be sensitive to the drugs. The differing potency of different drugs to inhibit depolarised induced contractions in different tissues, gave rise to the idea of tissue selectivity of calcium antagonists (Godfraind & Miller, 1986). The selectivity maybe a property of the compound or maybe a consequence of variation in physiological responses of tissues to stimuli e.g. dihydropyridines may not distinguish between calcium

channel types in the absence of depolarisation of the membrane.

Specific and high affinity binding sites for dihydropyridines and phenylalkylamines have been identified in smooth muscle, cardiac muscle and brain with radioligand binding studies (Glossmann *et al.*, 1982). The binding sites differentiate between the enantiomers of the dihydropyridines and between the stereoisomers of verapamil and D600. Calcium antagonists from one structural group could displace calcium antagonists from another group, but not as well as those from the same series (Glossmann *et al.*, 1982). From these type of results, it is suggested that there are separate binding sites for the different drug types and that these sites may be linked. (Triggle *et al.*, 1986).

Results from binding studies and electrophysiology suggest that the drug binding sites are localised in different parts of the channel. Verapamil and D600 are thought to act on the inner mouth of the calcium channel to inhibit calcium entry, having entered the channel while it was in the open state (Hescheler *et al.*, 1982). The dihydropyridine site of action has not been properly resolved. It is thought to be situated on the outer mouth of the channel, as the effect of the dihydropyridines increases with their lipophilicity (Kokubun & Reuter, 1984). However the divalent cation dependence of dihydropyridine binding (Glossmann *et al.*, 1982), suggests an association between ligand and metal ion

binding sites which indicates a more intracellular binding site for the drug (Hess & Tsien, 1984). Blockade of channels by dihydropyridine type antagonists reduces peak inward current and speeds its decay, which has been interpreted as the drug effecting open channels (Lee & Tsien, 1983). However, other work suggests that calcium channel antagonists may show preferential binding to inactivated channels (Pelzer *et al.*, 1982).

The dihydropyridines and phenylalkylamines are thought to have their antagonistic or agonistic effect by modulating the gating mechanism of the channels. It is thought that the binding of the drugs does not simply plug the the pore, but favours one or more of the modes of gating outlined previously. A dihydropyridine antagonist would promote mode 0 and a dihydropyridine agonist would favour mode 2. Using nimodipine (antagonist) and Bay K 8644 (agonist), this has been shown to be the case (Hess *et al.*, 1984). D600 (a phenylalkylamine antagonist), also appears to favour mode 0 and also tends to shorten individual openings within mode 1 (Fox *et al.*, 1986).

The inhibitory effect of the drugs is reinforced by inactivation of the calcium current. For example, prolonged depolarisations, which cause inactivation of channels, strongly promotes the inhibitory effect of nisoldipine. Other dihydropyridines act to slow recovery from inactivation following a depolarising pulse (Hess *et al.*, 1984).

1.12. Different types of calcium channels.

It has recently become apparent that there are several types of voltage-dependent calcium channels, each one with different properties. Multiple channel types have been identified in various neuronal tissues and muscle tissue (Nilius *et al.*, 1985; Nowycky *et al.*, 1985) and have been distinguished between by measuring the calcium current through several or single patch clamped calcium channels. From differences in conductance, three types, termed the L,T and N type calcium channels have been identified. The L-type channel has repeated openings which produce currents of relatively long duration (20 ms). The T-type channel, which can be activated by smaller depolarisations than L-type channels, produce transient inward currents (1 ms). N-type channels need very strong depolarisations to activate them and have neither of the inward current patterns seen for L and T-type channels. All three types of channels are found in neuronal cells, but only the L and T-type are found in cardiac and skeletal muscle (Reuter, 1986).

Apart from differences in activation and inactivation properties, the channel types can be distinguished on their sensitivity to various pharmacological agents. β -adrenoreceptor agonists seem only to effect L-type channels. The L-type channel is also more sensitive to organic drugs (dihydropyridines, phenylalkylamines, benzothiazepines and

diphenylbutylpiperidines) (Galizzi *et al.*, 1986; Glossmann & Striessnig, 1988) than the T-type, although the T-type can be inhibited by gallopamil and cinnarizine. N, L and T-type channels in neurons can be blocked by omega-conotoxin. The toxin reversibly inhibits the T channel from heart but has no effect on smooth or skeletal muscle channels (Miller, 1987; McCleskey *et al.*, 1987). As the dihydropyridines have more of an effect on heart and smooth muscle than in the nervous system and because only the L-type channels are sensitive to these drugs, it is suggested that the L-type channel is more abundant in heart and smooth muscle.

Each of the channel types may serve a particular function. It has been hypothesised that T-type channels could be involved in the rhythmic firing of neurons and specialised cardiac cells (Nowycky *et al.*, 1985; Bean, 1985). L-type channels may be important in contractile activation of cardiac and smooth muscle (Reuter, 1986). N-type channels are probably involved in neuronal calcium uptake and neurotransmitter release (Reynolds *et al.*, 1986; Dooley *et al.*, 1987).

1.13. The structure of voltage-dependent calcium channels.

Calcium channel antagonists have been used to structurally characterise voltage-dependent L-type calcium channels. (N-type channels have recently begun to be characterised using omega-conotoxin but as yet, no

structural information is available on T-type channels). Most of the information about the structural composition of the L-type channel comes from purification studies with either guinea-pig or rabbit skeletal muscle, using labelled dihydropyridines as a probe for the purified channel proteins either by direct binding assays or irreversible photoincorporation (Curtis & Catterall, 1984; Borsotto *et al.*, 1985; Striessnig *et al.*, 1986b, 1987). Some data is available on L-type channels in cardiac tissue (Cooper *et al.*, 1987). Skeletal muscle was used to purify the L-type channel as it was found to be the richest mammalian source of dihydropyridine and phenylalkylamine sensitive calcium channels, with binding site densities of 50-80 pmol/mg protein (Ferry & Glossmann, 1982a). The drug receptor sites are located on the transverse tubule membranes (Fosset *et al.*, 1983; Glossmann *et al.*, 1983).

After detergent solubilisation and purification, the skeletal muscle calcium antagonist receptor was shown to be composed of 5 subunits; α_1 , α_2 , β , γ , δ . This has been shown to be a functional calcium channel by reconstituting the purified calcium antagonist receptor into phosphatidylcholine vesicles and measuring uptake of $^{45}\text{Ca}^{2+}$ or $^{133}\text{Ba}^{2+}$. In the presence of the dihydropyridine (+)-PN200-110 and phenylalkylamine verapamil, uptake was blocked and Bay K 8644, (a dihydropyridine agonist), increased channel activity (Curtis & Catterall, 1986).

The α_1 subunit has an apparent molecular mass of 155-200 kDa (under reducing and non-reducing SDS PAGE conditions) and binds monoclonal and polyclonal antibodies that do not recognise the α_2 subunit (Takahashi *et al.*, 1987; Sharp *et al.*, 1987). Photoaffinity labelling with [^3H] azidopine (a DHP) and [^3H] LU 49888, (an analog of D888), indicate that the α_1 subunit contains the receptor sites for dihydropyridines and phenylalkylamines (Ferry *et al.*, 1984b, 1986; Striessnig *et al.*, 1986a, 1987; Vaghy *et al.*, 1987). The subunit has N-glycosylation sites but little or no carbohydrate attached and a hydrophobic affinity probe indicates that it has multiple transmembrane segments (Tanabe *et al.*, 1987; Takahashi *et al.*, 1987; Sharp *et al.*, 1987). The α_1 subunit is thought to be the main functional subunit of the dihydropyridine sensitive L-type channel and may act as both the voltage-activated calcium channel and the voltage sensor (Tanabe *et al.*, 1987; Agnew, 1987).

The α_2 subunit has an apparent molecular mass of 135-150 kDa after reduction of disulfide bonds. It appears to be glycosylated (Leung *et al.*, 1987) and has no significant transmembrane hydrophobic domains. The subunit is linked to a glycoprotein of molecular mass 24-29 kDa called the δ subunit, which increases the apparent size of the α_2 subunit to 175 kDa when disulfide bonds are intact. Since the $\alpha_2\delta$ complex is heavily glycosylated

and has few transmembrane segments, it may be exposed at the extracellular surface.

The β and γ subunits are peripherally associated with the α_1 subunit. The β subunit is neither glycosylated, nor labelled by hydrophobic probes (Takahashi *et al.*, 1987) and so may be attached to the intracellular surface of the calcium channel complex. The γ subunit is both labelled by hydrophobic probes and is glycosylated and so may be a intracellular and/or extracellular component of the channel. Fig.1.4. shows a model of dihydropyridine sensitive calcium channel subunit structure as proposed by Takahashi *et al.*, (1987).

Despite the fact that the α_1 subunit contains much of the functional activity of the channel, the other subunits do appear to be a vital part of the calcium channel complex, although this has been questioned. One piece of evidence that all the subunits are needed for a functional channel is shown by attempts to break the non-covalent bonds that hold the subunits together. If broken, reversible dihydropyridine binding is lost, which suggests that the non- α_1 components stabilise the channel in a high affinity conformation.

The α_1 subunit from rabbit skeletal calcium channels has been cloned (Tanabe *et al.*, 1987) and has some homology with the voltage-dependent sodium channel. In particular there is homology between transmembrane segments which are thought to play an important role in

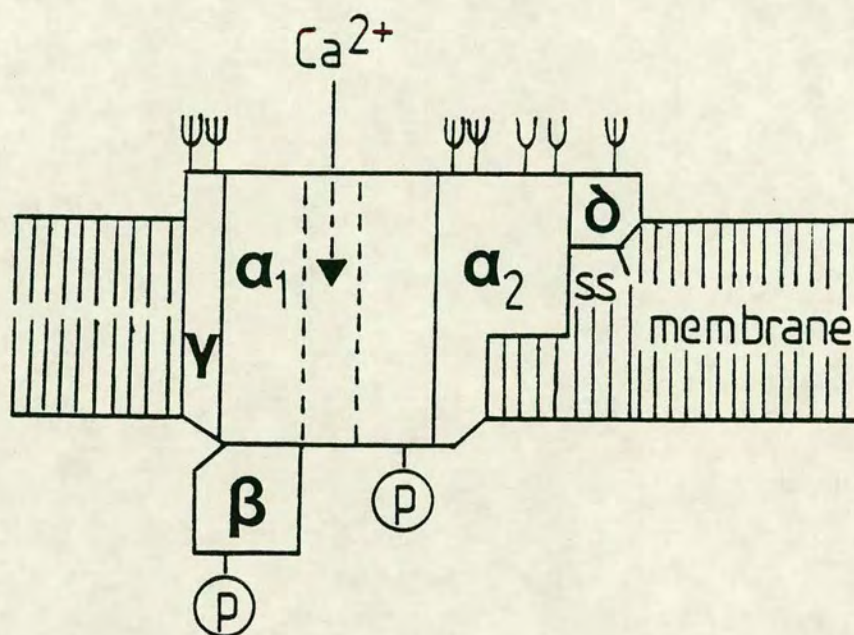


Fig.1.4. Model of the subunit structure of the voltage-dependent L-type calcium channel (after Takahashi *et al.*, 1987).

The model shows the proposed structure of the voltage-dependent (dihydropyridine-sensitive) calcium channel from rabbit transverse tubule membranes. P indicates the sites of cAMP-dependent phosphorylation. Ψ and Υ indicate sites of glycosylation. SS denotes the disulphide bonds linking δ to α_2 .

ion transport and voltage-dependent gating in the sodium channel. These regions are highly conserved in the calcium channel structure, suggesting that the α_1 subunit is important in ion transfer and voltage sensing. Also seen are regions resembling parts of calcium binding proteins, which fits in with electrophysiological and biochemical data postulating two divalent cation binding sites within the channel pore (Hess & Tsien, 1984).

1.14. Regulation of voltage-dependent calcium channels.

Voltage-dependent calcium channels are thought to be regulated by neurotransmitter-mediated phosphorylation and dephosphorylation events. Electrophysiological and $^{45}\text{Ca}^{2+}$ studies have shown that cAMP and cAMP dependent protein kinase can influence the opening of calcium channels in cardiac and skeletal muscle (Schmid *et al.*, 1985; Flockerzi *et al.*, 1986a,b). Regulation of the dihydropyridine sensitive channel by cAMP-dependent phosphorylation may involve phosphorylation of the calcium channel itself or associated proteins which regulate channel activity. Experimental evidence suggests that two components of the channel itself i.e. the α_1 and β subunits are sites of cAMP-dependent phosphorylation (Curtis & Catterall, 1985; Imagawa *et al.*, 1986). Other studies have shown that the α_1 subunit in skeletal muscle is the major substrate for cAMP-dependent protein kinases (Hosey *et al.*, 1986,1987; Takahashi *et al.*, 1987) and calcium/calmodulin-dependent protein kinase. Evidence

suggests that the α_1 subunit appears to be multiply phosphorylated at distinct sites by both kinases (O'Callahan, 1988). Therefore as well as being capable of forming an ion channel and acting as a voltage sensor, the α_1 subunit also contains sites necessary for neurotransmitter regulation via phosphorylation. Other studies have suggested that calcium channels may be regulated by phosphorylation of the β subunit by protein kinase C (Nastainczyk *et al.*, 1987; Galizzi *et al.*, 1987).

Electrophysiological work indicates that G proteins can also modulate T,L and N-type channel function in neuronal cells (Scott & Dolphin, 1987; Hescheler *et al.*, 1987). G-proteins and their subunits are also thought to regulate L-type channels in muscle by mediating neurotransmitter and hormone action (Yatani *et al.*, 1987). However these observations have not been confirmed using purified calcium channel preparations.

1.15. Calcium channels in plants.

If plant cells are to use calcium ions as a second messenger, the entry of these ions into the cell needs to be rapid and regulated. The plasma membrane is fairly impermeable to calcium and so movement of calcium ions into the cell is slow. To take advantage of the steep electrochemical gradient that exists for calcium suggests that plant cells have calcium channels as these would allow the controlled but fast entry of calcium ions into

the cell. This would cause a rise in cytosolic calcium which could then act as a signal. However, evidence for the existence of calcium channels in plant membranes is limited. -

Various pieces of experimental evidence point to the existence of calcium channels in plants. Chloroplast movement in the alga *Mougeotia* is a light-dependent, calcium-mediated response, which occurs fairly rapidly. The speed of the response may be explained by calcium channels which would allow rapid inflow of ions to initiate chloroplast movement (Wagner *et al.*, 1984). This suggestion has been supported by electrophysiological experiments with *Chara*, that indicate a calcium action potential-dependent increase of cytoplasmic calcium which inhibits cytoplasmic streaming (Hayama *et al.*, 1979; Williamson & Ashley, 1982; Kikuyama & Tazawa, 1983).

Electrophysiological studies on growing rhizoid cells of *Fucus serratus* also suggest the presence of calcium channels. Experiments on this system demonstrate the presence of a longitudinal gradient of cytosolic calcium in the rhizoid cell, which appears to be maintained by preferential calcium influx in the region of the growing tip (Brownlee & Wood, 1986). From this work it was postulated that the calcium influx is via calcium channels and the gradient the influx supports may be involved in cell polarity.

Data from experiments using $^{45}\text{Ca}^{2+}$ flux measurements also point to the presence of voltage controlled calcium

channels in plant membranes. Results from such experiments indicated that influx of $^{45}\text{Ca}^{2+}$ into non-growing corn root tissue was increased as a result of actions such as cutting, chilling and heating or chemicals such as cyanide, which are known to depolarise the cell membrane. Calcium flux was decreased by actions such as washing or chemicals such as fusicoccin which hyperpolarise the membrane. (Rincon & Hanson, 1986).

Other studies using calcium antagonists also suggest that calcium channels exist in plant cells. As already mentioned, labelled antagonists such as nifedipine and verapamil, have been used to identify binding sites in various animal tissues, so indicating the presence of a calcium channel (Glossmann *et al.*, 1982; Galizzi *et al.*, 1984a,b). The same approach has been used for various plant tissues. Hetherington & Trewavas (1984) demonstrated [^3H] nitrendipine binding to pea shoot membranes and Andrejauskas *et al.* (1985) have demonstrated specific binding of [^3H] verapamil to zucchini membranes. More recently specific binding of [^3H] verapamil to membrane fractions of *Chlamydomonas reinhardtii* has been identified (Dolle & Nultsch, 1988).

Polar growth of lily pollen tubes has been shown to be affected by nifedipine, causing irregular growth patterns, which suggests the presence of nifedipine sensitive calcium channels in pollen tubes (Reiss & Herth, 1985). Similarly verapamil and LaCl_2 have been found to effect morphogenesis in *Microsterias* (Lehtonen,

1984) and calcium antagonists have also been shown to block cytokinin induced bud formation in *Funaria* (Saunders & Hepler, 1983).

Although plant membranes have binding sites for antagonists and cell functions known to be mediated by calcium are effected by antagonists, it has only recently been demonstrated that calcium antagonists can block calcium entry into a plant cell (Graziana *et al.*, 1987). In these experiments $^{45}\text{Ca}^{2+}$ uptake into carrot protoplasts in the presence of calcium channel inhibitors was studied. The results indicated that antagonists of the phenylalkylamine and diphenylbutylpiperidine series, as well as bepridil, could inhibit $^{45}\text{Ca}^{2+}$ flux into carrot protoplasts. The dihydropyridines did not have an inhibitory effect and specific binding sites for the dihydropyridine [^3H](+)-PN 200-110 could not be identified. A high density of [^3H]-(-)-D888 binding sites were found. The results from this work strongly suggest that the D888 binding site does represent the calcium channel in plant membranes and provides further evidence for the existence of calcium channels in plant cells.

1.16. Summary.

Calcium channels appear to be present in plant membranes. Apart from knowledge of their possible existence, very little else is known about them in plants. Up until now there have been no reported attempts to purify the plant calcium channel and so nothing about

its structure has been elucidated. Any similarities between plant and animal channels in terms of structure, have so far been speculative, but it is known that the protein structures of antagonist receptors in plant and animal membranes must have some differences, as monoclonal and polyclonal antibodies (Schmid *et al.*, 1986a,b; Vandaele *et al.*, 1987; Cooper *et al.*, 1987), developed for dihydropyridine and phenylalkylamine receptors in skeletal muscle, do not cross react with plant membranes (personal communication from M. Lazdunski).

In order to increase our understanding of calcium channels in plant cells and to obtain some information about the structure and possible regulation of plant calcium channels, an investigation was carried out using the channel antagonist [^3H] verapamil. Binding studies were performed and a method developed to solubilise and partially purify the verapamil binding protein, (and possibly therefore the whole calcium channel), from maize coleoptile membranes. The following sections of this thesis presents the results of this investigation.

CHAPTER 2.

MATERIALS AND METHODS.

2.1. Plant material.

Maize seeds, (*Zea mays* cv. Leader), were a generous gift from the National Institute of Agricultural Botany, Cambridge, U.K. Zucchini seeds, (*Cucurbita pepo*) were a kind gift from Dr. M.A.Venis. All seeds were stored in the dark at 20°C until required for use.

2.2. Radiochemicals.

[N-methyl-³H] verapamil hydrochloride, with a specific activity of 68.8 Ci/mmol was obtained from New England Nuclear, Boston, Massachusetts, U.S.A. (-)-[N-methyl-³H] desmethoxyverapamil hydrochloride, with a specific activity of 85 Ci/mmol, was obtained from Amersham International Plc, Amersham, Buckinghamshire, U.K. [³H] LU 49888 (Azidopamil), with a specific activity of 79.65 Ci/mmol, was a generous gift from Knoll AG, West Germany. [γ -³²P] Adenosine triphosphate was obtained from Amersham International Plc at a specific activity of 4995 Ci/mmol. ⁴⁵Calcium chloride in aqueous solution with a specific activity of 10-40 mCi/mg calcium, was obtained from Amersham International Plc. All radiochemicals were stored at -20°C.

2.3. Chemicals.

Unlabelled (+) or (-)-verapamil hydrochloride was

obtained from Sigma, Poole, Dorset. Unlabelled (-)-desmethoxyverapamil hydrochloride was from Amersham International Plc. Unlabelled methoxyverapamil was a generous gift from Dr. M.A. Venis. Nifedipine was obtained from Bayer U.K. Haywards Heath, Sussex. U.K. All other chemicals were of the highest grade available and were obtained from either Sigma, Poole, Dorset, U.K. or BDH Chemical Company, Poole, Dorset, U.K.

2.4. Centrifuges.

Superspeed-65 ultracentrifuge and Micro-Centaur microfuge were from MSE Scientific Instruments, Manor Royal, Crawley, U.K. The Sorvall RC-5B was from the Du Pont Company, Biomedical products division, Newton, Connecticut, U.S.A.

2.5. Liquid Scintillation Counting.

The scintillation counter was a Kontron Intertechnique from Kontron Instruments Ltd, Hertfordshire, U.K. Scintillation fluid for aqueous samples was prepared from Toluene (1L), Triton x-100 (0.5L), Butyl-PBD (7g). Scintillation fluid for non-aqueous samples was prepared from Toluene (1L) and Butyl-PBD (4g).

2.6. pH Meter.

The pH meter, EIL model 7010 was from Mackay and Lynn Ltd, Edinburgh, U.K.

2.7. Preparation of Plant Tissue.

Maize seeds were first washed to remove fungicide ^{to} and then imbibed in fresh water for two [^]three hours. The seeds were then sown in trays of moist vermiculite and allowed to grow in the dark at 25⁰C. After 5 days the coleoptiles, including the leaf rolls, (unless indicated otherwise), were harvested and collected on ice. When maize root tissue was required, maize seeds were sown on wet tissue and grown under the same conditions. Roots were harvested after 5 days. Zucchini seeds were grown in vermiculite as above and the hypocotyls harvested after 5 days.

2.8. Preparation of Crude Membrane Fractions.

All preparative procedures were carried out at 4⁰C, unless stated otherwise. Freshly harvested tissue was chopped with a razor blade on a perspex sheet and then homogenised in 2 ml per gram fresh weight of 50 mM Tris, 3 mM EDTA buffer, pH 7.5 with HCl, in a pestle and mortar. The homogenate was then filtered through one layer of nylon cloth (pore size 100 μ m) and the resulting suspension centrifuged at 4000 g for 20 min in a Sorvall SS 34 rotor using a Sorvall RC-5B centrifuge. The pellet was discarded and the supernatant re-centrifuged at 50,000 g for 45 min as above. This yielded the crude membrane pellet which was resuspended in 50 mM Tris, 50 mM Mops buffer, pH 7.5 with HCl, using a glass-Teflon homogeniser. The resuspension was termed as the the crude

membrane fraction or preparation.

2.9. Solubilisation of the Crude Membrane Fractions.

The crude membrane preparations was solubilised in a solubilisation buffer containing 50 mM Tris, 50 mM Mops, pH 7.5 with HCl, \pm 20% sucrose or glycerol, plus varying concentrations of one of the following detergents:- CHAPS, octyl glucoside and Triton x-100. After a 30 min incubation at 4⁰C, the mixture was centrifuged at 100,000g for 1 hour in a MSE Superspeed-65 ultracentrifuge, using a 10 x 10_{ml} MSE aluminium fixed angle rotor. The resulting supernatant contained the solubilised proteins.

In the final solubilisation procedure the crude membrane preparation was incubated at a protein:detergent ratio of 1:14 (w/w) in 0.5% CHAPS, 20% sucrose, 50 mM Tris, 50 mM Mops buffer, pH 7.5 with HCl, for 30 min at 4⁰C and then centrifuged for 1 hour at 100,000 g as above. The solubilised crude membrane was termed as the solubilised membrane fraction or CHAPS extract.

2.10. Phase Partitioning of Crude Membrane.

The method employed for plasma membrane isolation was based on that by Yoshida *et al.* (1983). Crude membrane was prepared as described previously and the crude membrane pellet resuspended in +NaCl buffer containing 0.5 M sorbitol, 15 mM Tris/maleate, 30 mM NaCl, pH 7.3 with KOH. Aliquots (500 μ l) of the

resuspension were layered onto polyethyleneglycol/Dextran phases to produce 4g phases with a final composition of 5.6% (w/w) each of polyethyleneglycol (approximate molecular weight of 3,350 Da) and Dextran (average molecular weight of 472,000 Da) in +NaCl buffer. The phases were mixed thoroughly by 30 inversions and left to settle for 30 min at 0°C. Brief centrifugation in a bench centrifuge was used to aid phase separation. Upper and lower phases were separated and diluted with 30 ml of -NaCl buffer, containing 0.5 M sorbitol, 15 mM Tris/maleate, pH 7.3 with KOH and centrifuged at 50,000 g for 1 hour in a Sorvall SS 34 rotor using a Sorvall RC-5B centrifuge. The resulting upper phase pellet is referred to as the plasma membrane enriched fraction and the lower phase as the residual membrane fraction.

2.11. Verapamil Binding Assays.

2.11.1. *Centrifugation Method.*

[³H] verapamil was added to the crude membrane resuspension (approximately 0.4 mg protein/1 ml), to give a final concentration of 0.3 nM. The resuspension was then divided into 1 ml cellulose acetate butyrate tubes, (Sorvall, Du Pont Biomedical Division, Newtown, Connecticut U.S.A.), which contained unlabelled verapamil at a range of concentrations. To determine total binding of [³H] verapamil, membrane resuspension was added to a tube containing an equivalent volume of distilled H₂O or methanol. The solutions were then incubated for 2 hours

at 20⁰C. The resuspensions were then centrifuged at 50,000 g for 30 min in Sorvall 1 ml tube adaptors in a Sorvall SS 34 rotor using a Sorvall RC-5B centrifuge. The supernatants were decanted and the tubes left to drain. The pellets were then washed carefully with distilled H₂O, left to drain and any adhering drops of liquid blotted with tissue. Bound radioactivity in the pellet was then recovered by resuspending the pellets in a total volume of 1.3 ml of distilled H₂O. The resuspension was placed in scintillation vials with 10 ml of aqueous scintillation fluid and counted in a scintillation counter. Non-specific binding was determined by incubating parallel samples with 100 µM unlabelled verapamil. The value obtained from these samples was subtracted from the total binding to give the specific binding of [³H] verapamil.

2.11.2. Filter Method.

In the final assay, referred to as the standard filter assay, [³H] verapamil was added to the crude membrane resuspension (0.5 mg/500 µl), solubilised membrane fraction (0.5-1.0 mg/500 µl) or column fraction to give a final concentration of 2.24 nM. Unlabelled verapamil or other compounds, at a range of concentrations, were then added to the final assay volume of 500 µl, as indicated in the figure legends. After incubation for 120 min at 20⁰C, the reaction was stopped by the addition of 3 ml of ice-cold wash buffer

containing 10% PEG 8000, 50 mM Tris, at pH 7.5 with HCl. The mixture was immediately filtered under vacuum through a GF/C filter (Whatman, Maidstone, Kent, U.K.), using a Millipore multifilter unit (Millipore, Bedford, Mass. U.S.A.). When solubilised fractions were assayed the filters were pre-treated with 0.3% polyethyleneimine (PEI) solution for at least 2 hours before use (Bruns *et al.*, 1983). The filters were then washed once with 3 ml of ice-cold wash buffer to remove any unbound label. The radioactivity retained on the filter was determined by liquid scintillation counting in 5 ml of aqueous scintillant for 10 min using a liquid scintillation counter. As described previously, non-specific binding was determined by incubating parallel samples with 100 μ M unlabelled verapamil and was subtracted from total binding, (obtained from assays containing [3 H] verapamil only), to give the specific binding of [3 H] verapamil. All assays were carried out in duplicate or triplicate and the results expressed as an average for each set of replicates. This assay method was also used in binding studies with [3 H] desmethoxyverapamil.

2.12. [3 H] LU 49888 Binding Assay.

In the standard binding assay, [3 H] LU 49888 was added to the crude membrane resuspension to give a final concentration between 0.1 nM - 18 nM as indicated in the figure legends. Unlabelled desmethoxyverapamil was added to an assay volume of 500 μ l to give a final

concentration of 100 μ M. Total binding was determined in parallel samples containing labelled antagonist only. After incubation for 120 min at 20⁰C in the dark, the reaction was stopped by addition of ice-cold wash buffer as before and bound radioactivity determined by the standard filter assay.

2.13. Column Chromatography.

All chromatography columns were prepared at 4⁰C and packed according to the manufacturers' recommended instructions. Unless indicated all column chromatography was carried out at 4⁰C. Where appropriate, fractions were collected using a 2070 Ultrorac 2 fraction collector (LKB instruments Ltd, Croydon, Surrey, U.K.). A single path UV-1 column monitor (Pharmacia), was also used in some of the column chromatography experiments. The following table (Table 2.1.), outlines the columns and column matrices used, plus their suppliers.

The standard column buffer, used for all the column chromatography experiments, contained 50 mM Tris, 50 mM Mops, 0.1% CHAPS, 20% Sucrose, pH 7.5 with HCl, unless stated otherwise.

[³H] verapamil binding in each column fraction was determined using the standard filter assay.

<u>Method.</u>	<u>Matrix.</u>	<u>Column support.</u>
<u>Anion exchange.</u>	DEAE	Pharmacia K9
	Bio-Gel A. ¹	I.D. 9 mm. Length 15 cm
<u>Gel filtration.</u>	Sephacryl	Bio-Rad Econocolumn
	S300/S400. ²	I.D. 15 mm. Length 30 cm
<u>Hydrophobic</u>	Phenyl	Pharmacia C10
<u>Interaction.</u>	Sepharose	I.D. 10 mm. Length 10 cm
	CL-4B. ³	
<u>Lectin affinity.</u>	Wheat Germ	-
	Agarose. ³	
	Concanavalin	-
	A-Sepharose 4B. ²	

Table 2.1. Chromatographic materials.

Column matrices and column supports were supplied by either:- 1. Bio-Rad, Watford, Herts, UK.

2. Pharmacia, Milton Keynes, Bucks, UK.

3. Sigma Chemical Company, Poole, Dorset, UK.

2.14. DEAE Ion Exchange Chromatography.

2.14.1. *Linear NaCl gradient elution.*

A 4 x 1 cm DEAE Bio-Gel A column was prepared and equilibrated with at least 10 bed volumes of column buffer. The bed volume of the column, determined using m^{2h} , was 3.14 ml. Approximately 10 ml of the solubilised membrane fraction, from approximately 50-100 g fresh weight of tissue, was loaded onto the pre-equilibrated column. After loading was complete, the column was washed with 10 ml of column buffer. The verapamil binding activity was then eluted using a linear gradient of 0-0.5 M NaCl in column buffer. The gradient was formed in gradient maker, (Botany Dept, Workshop). 2 ml fractions were collected, at a flow rate of 1 ml/1.5 min and assayed for [3H] verapamil binding. In a parallel experiment the elution gradient was applied to the DEAE column alone. 2 ml fractions were collected, placed in aluminium coated cuvettes, (Bio-Rad, Watford, Herts, U.K.), and a conductivity reading taken for each fraction using a M-102BZ conductivity meter, (Maplin electronic supplies, Rayleigh, Essex, U.K.). Conductivity readings were also taken for samples containing 0.1 M, 0.2 M, 0.4 M & 0.5 M NaCl only.

2.14.2. *Step NaCl gradient elution.*

A 4 x 1 cm DEAE Bio-Gel A column was prepared as described previously. Approximately 10 ml of solubilised membrane fraction was loaded onto the column and the

column then washed with 10 ml of column buffer. The verapamil binding activity was eluted from the column using 10 ml of 0.1 M and 10 ml of 0.2M NaCl in column buffer. Fractions of 1 ml were collected and assayed for [³H] verapamil binding.

2.14.3. Final DEAE elution protocol.

In the final purification procedure, the DEAE ion exchange step was carried out as follows. Solubilised membrane fraction from approximately 100 g fresh weight of tissue was loaded onto a 4 x 1 cm DEAE Bio-Gel A column, which had been pre-equilibrated with column buffer. The ratio of sample to matrix was 5:1 (v/v). After loading the sample, the column was washed with an equal volume of column buffer. Elution with 0.15 M in column buffer yielded the verapamil binding activity in the first 8 ml of the eluate. This was collected, aliquots assayed directly for verapamil binding and the eluate then quick-frozen in liquid nitrogen and stored at -80°C until required for further purification. A fresh DEAE column was prepared at the start of each new purification procedure.

2.15. Gel Filtration Chromatography.

2.15.1. Sephacryl S300 column.

A Sephacryl S300 column, (fractionation range 1×10^4 - 1.5×10^6 Daltons), was prepared and equilibrated with 5 bed volumes of column buffer. The bed volume of

the column, determined using nr^{2h} , was 9.62 ml. 0.5 ml of peak DEAE fraction was then loaded onto the column and eluted with column buffer. 1 ml fractions were collected, at a flow rate of 1 ml/min. Each fraction was assayed for [3H] verapamil binding using the standard filter assay. The effects of including 0.2 M NaCl, 1.0 M NaCl or 1 mM DTT in the column buffer were also investigated.

The void volume of the column was determined separately using Blue Dextran 2000 monitored at A_{280} using a UV Spectrophotometer (Pye Unicam, Cambridge, U.K.).

2.15.2. *Sephacryl S400 column.*

In the final purification procedure a Sephadryl S400 column, (fractionation range $2 \times 10^4 - 8 \times 10^6$), with a bed volume of 38.8 ml, was prepared and equilibrated with column buffer at a flow rate of 0.5 ml/min. 2 ml of pooled peak verapamil binding fraction from the DEAE column, was loaded onto the wet bed surface of the column and eluted with column buffer. Fractions of 1 ml were collected and aliquots assayed for verapamil binding activity. Fractions with the highest binding activity were pooled prior to further purification. As above, the void volume of the column was determined using Blue Dextran 2000.

At the end of each column run, the S300 or S400 columns were washed with 2 bed volumes of column buffer

and the column then stored in 0.02% sodium azide until required.

2.16. Hydrophobic Interaction Chromatography.

2.16.1. *Phenyl Sepharose column buffers.*

Three main buffers, plus variations of these, were used in a series of experiments in order to find the optimum phenyl Sepharose column elution protocol;

Low salt Buffer. 50 mM Tris, 50 mM Mops, 20% sucrose, 0.1% CHAPS, 0.1 mM CaCl_2 , pH 7.5 with HCl.

High Salt Buffer. 50 mM Tris, 50 mM Mops, 20% sucrose, 0.1% CHAPS, 1 M $(\text{NH}_4)_2\text{SO}_4$, 0.1 mM CaCl_2 , pH 7.5 with HCl.

Low Detergent/Sucrose Buffer.

50 mM Tris, 50 mM Mops, 8.5% (0.25 M) sucrose, 0.01% CHAPS, 0.1 mM CaCl_2 , 1 M $(\text{NH}_4)_2\text{SO}_4$, pH 7.5 with HCl.

2.16.2. *Phenyl Sepharose column elution protocol. Basic Method.*

A 4 x 1 cm phenyl Sepharose Cl-4B column, connected to a UV.1 column monitor, was prepared and equilibrated with 10 bed volumes of one of the three basic buffers. Approximately 4 ml of pooled peak verapamil binding fractions from the DEAE column were either adjusted to

1 M $(\text{NH}_4)_2\text{SO}_4$ and 1 mM CaCl_2 or 1 mM CaCl_2 only and loaded onto the column. The column was then washed with the same equilibration buffer, until the A_{280} trace returned near to baseline. After washing, the column was eluted by the removal of salt, calcium or Tris/Mops from one of the three basic buffers, as indicated in the figures. The A_{280} trace was allowed to return near to baseline in between subsequent elution steps. Fractions of 4 ml were collected and 2 ml was assayed for [^3H] verapamil binding activity using the standard filter assay.

Using this basic method a number of elution protocols were experimented with in order to find the optimum elution procedure for the phenyl Sepharose column. The details of the different protocols are shown in Table 2.2.

2.16.3. Final phenyl Sepharose column elution protocol.

In the final purification procedure, pooled peak verapamil binding fractions from the Sephacryl S400 column were adjusted to 1 M $(\text{NH}_4)_2\text{SO}_4$, 1 mM CaCl_2 . These were then loaded onto a 2 x 1 cm column of phenyl Sepharose CL-4B which had been equilibrated with 10 bed volumes of high salt buffer. After washing the column with up to 18 ml of high salt buffer, the column was eluted with 18 ml each of the following series of buffers: low salt buffer, low calcium buffer and minus Tris/Mops buffer. Fractions of 2 ml were collected and

PROTOCOL NUMBER.	SAMPLE ADJUSTMENT.	COLUMN LOAD & WASH CONDITIONS.	ELUTION STEPS.				
		1.	2.	3.	4.	5.	6.
1.	1 mM CaCl ₂	Low salt	Low Ca ²⁺	Minus Tris/Mops	Minus Ca ²⁺	H ₂ O	-
2.	1 mM CaCl ₂ 1M (NH ₄) ₂ SO ₄	High salt	Low salt	Low Ca ²⁺	Minus Tris/Mops	Minus Ca ²⁺	H ₂ O
3.	1 mM CaCl ₂ 1 m (NH ₄) ₂ SO ₄	Low detergent/ Low sucrose	Low salt	Minus Tris/Mops (low detergent/sucrose)	Minus Tris/Mops (high detergent/sucrose)	-	-
4.	1 mM CaCl ₂ 1 M (NH ₄) ₂ SO ₄	High salt	Low salt	Minus Tris/Mops (low detergent/sucrose)	Minus Tris/Mops (high detergent/sucrose)	-	-
5.	1 mM CaCl ₂ 1 M (NH ₄) ₂ SO ₄	High salt	Low salt	Low Ca ²⁺	Minus Tris/Mops	-	-

Table.2.2. Phenyl Sepharose column elution protocols.

the first four 2 ml fractions of each elution step were assayed for [^3H] verapamil binding.

2.17. Lectin Affinity Chromatography.

2.17.1. Con-A Sepharose elution protocol.

Approximately 1 ml of solubilised membrane fraction was applied to 300 μl of Con-A Sepharose contained in an eppendorf tube. The Con-A had previously been washed with 5 ml of Con-A column buffer containing 0.02 M Tris-HCl, 0.5 M NaCl, 0.5 mM CaCl_2 , 0.5 mM MnCl_2 , 0.1% CHAPS, 20% sucrose, pH 7.5. The Con-A plus the sample were then incubated for 2 hours at 4°C and then centrifuged at 11,600 g for 5 min. The supernatant, referred to as the load supernatant (LSN) was removed and assayed for [^3H] verapamil binding. The Con-A was then washed three times with 1 ml of Con-A column buffer and centrifuged after each wash. The wash supernatants (WSN) were pooled and assayed for [^3H] verapamil binding. The Con-A was then sequentially eluted with with 1 ml each of 0.1 M, 0.2 M, 0.3 M, 0.4 M and 0.5 M α -methyl mannoside in Con-A column buffer. Each concentration of elution buffer was applied to the Con-A in turn and the mixture then centrifuged. The supernatants from each elution step (ESN) were assayed for [^3H] verapamil binding. The standard filter assay for [^3H] verapamil binding was used throughout.

2.17.2. Wheat Germ Agarose column elution protocol.

Approximately 1 ml of solubilised membrane fraction was applied to 300 μ l of wheat germ agarose (WGA) contained in an eppendorf tube. The WGA had previously been equilibrated with 5 ml of standard column buffer. The WGA plus the sample were then incubated for 2 hours at 4⁰C and then centrifuged at 11,600 g for 5 min. The LSN was then assayed for [³H] verapamil binding. The WGA was then washed with standard column buffer as described above and the pooled WSN assayed for [³H] verapamil binding. Elution of the WGA was carried out using 1 ml of 100 mM n-acetyl-D-glucosamine in standard column buffer. This was applied to the WGA and the mixture then centrifuged as before. The ESN was assayed for [³H] verapamil binding. The standard filter assay for [³H] verapamil binding was used throughout.

2.18. SDS Polyacrylamide Gel Electrophoresis.

2.18.1. Equipment.

Sodium-dodecyl-sulphate (SDS) polyacrylamide gel electrophoresis was carried out using vertical slab gels. Electrophoresis equipment was obtained from the Botany Dept. workshop or Dual vertical slab gel electrophoresis equipment from Bio-Rad, Watford, Herts, U.K. was employed. The Mighty Small vertical slab gel unit was obtained from Hoeffer Scientific instruments, San Francisco, California, U.S.A..

2.18.2. Gel Preparation.

Slab gels with dimensions of 16 x 16 cm or 8 x 6 cm and 1.5 mm thickness, were prepared from stock solutions which were made up as follows:

30% Acrylamide solution: 29.2% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide. Stored at 4°C.

Resolving gel buffer (RGBx4): 1.5 M Tris-HCl pH 8.8, 0.4% SDS. Stored at 4°C.

Stacking gel buffer (SGBx4): 0.5 M Tris-HCl pH 6.8, 0.4% SDS. Stored at 4°C.

10% AMPS. Stored at 4°C and made up fresh for each gel.

TEMED, obtained from Sigma, Poole, Dorset.

The stacking gel contained 5% (w/v) acrylamide, 0.13% (w/v) bis-acrylamide, 125 mM Tris-HCl pH 6.8, 0.1% (w/v) SDS. This was degassed under vacuum and then 0.03% (w/v) AMPS and 0.1% (v/v) TEMED were added to polymerise the gel.

The resolving gel contained 12% (w/v) acrylamide, 0.32% (w/v) bis-acrylamide, 375 mM Tris-HCl pH 8.8, 0.1% (w/v) SDS, degassed under vacuum and then 0.015% (w/v) AMPS and 0.05% (v/v) TEMED were added to polymerise the gel. Where necessary, volumes were adjusted to create resolving gels containing 10% or 7% acrylamide.

2.18.3. Sample Preparation.

Samples for SDS-PAGE were denatured in an equal volume of sample buffer containing 4.7 % (w/v) SDS, 10%

(v/v) 2-Mercaptoethanol, 20% (w/v) glycerol, 125 mM Tris pH 6.8. Bromophenol blue (0.02%) at 15 μ l/100 μ l sample was added as a tracking dye. The samples were then heated in a Tecam DB-3 dri-block (Techne, Cambridge, U.K.), for 10 min at 100°C and allowed to cool. The samples of 100 μ l were loaded into the gel slots using a Hamilton syringe.

Phenyl Sepharose fractions were concentrated using a Speed-Vac centrifugal evaporator (Hicksville, New York, U.S.A.), before samples for gel electrophoresis were prepared. This was done because the amount of protein in column fractions from the phenyl Sepharose column was very low and could not be detected on a gel by silver staining, unless concentrated first.

2.18.4. Running Conditions.

The gels were run at 20°C in electrode buffer containing 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, at 300 Volts, 20 mA (constant current) for large gels or 150 Volts, 18 mA (constant current) for Mighty Small gels.

2.18.5. Molecular Weight Markers.

Myosin (205 kDa), B-galactosidase (116 kDa), Phosphorylase B (97.4 kDa), Bovine Serum Albumin (66 kDa), Ovalbumin (45 kDa) and Carbonic Anhydrase (29 kDa) were used as molecular weight markers. All markers were obtained from Sigma, Poole, Dorset, U.K.. A stock solution containing the molecular weight markers at 1-2



mg/ml, plus an equal volume of sample buffer and Bromophenol blue was prepared as described previously. When required approximately 10 μ l of the stock was diluted 1:1 with sample buffer, heated at 100°C for 10 min, allowed to cool and then loaded into the gel slots as before.

2.19. Visualisation of proteins in polyacrylamide gels.

2.19.1. Coomassie blue staining.

Polyacrylamide gels were fixed and stained for 30 min in 0.2% (w/v) Coomassie Brilliant Blue R in 50% (v/v) methanol and 7% (v/v) acetic acid. The gel was then destained in 25% (v/v) ethanol, 8% (v/v) acetic acid for 1 hour. Background staining was removed by an overnight wash in storage solution containing 5% (v/v) methanol, 7% (v/v) acetic acid. Gels were then dried down under vacuum and mild heat onto Whatmann 3MM chromatography paper.

2.19.2. Silver Staining.

Silver staining was carried out essentially according to the method of Morrissey (1981), using a silver staining kit from Sigma, Poole, Dorset, U.K.. Deionised distilled water (d.d.H₂O) was used for all silver staining solutions. After electrophoresis the gel was fixed in 3 stages. 1. 50% (v/v) methanol, 10% (v/v) acetic acid for 30 min, followed by, 2. 30 min in 5% (v/v) methanol, 7% (v/v) acetic acid followed by, 3. 10% (v/v) glutaraldehyde for 30 min. The gel was then washed

in several changes of d.d.H₂O over a period of 2 hours, followed by a 30 min wash in 5 µg/ml DTT. The gel was then transferred to the silver equilibration solution and gently agitated for 30 min, followed a rapid wash in d.d.H₂O. The water was then replaced by developer solution for 5-8 min and left until the bands began to appear on the gel. After sufficient development time, (5 min), the developer solution was replaced by a stop solution of 1% (v/v) acetic acid. The gel was then rinsed 3 times for 10 min each time with a large volume of d.d.H₂O. If necessary, reducer solution was added to remove background staining. The gel was then left in d.d.H₂O and a small amount of glycerol overnight and dried down as described previously.

2.20. Photography.

Gels were photographed using Kodak Technical Pan film 2415, (Eastman Kodak Company, Rochester, N.Y. U.S.A.). Films were developed in Kodak HC-110 developer for 5 min, the development stopped in 1% acetic acid for 2-3 min and the film then fixed for 5-10 min in Ilford Hypam fixer (Ilford Ltd. Moberly, Cheshire, U.K.). Prints were made on Ilford Ilfospeed photographic paper No.3. and developed in Ilford Ilfospeed paper developer, the development stopped in 1% acetic acid for 1-2 min and the print then fixed in Ilford Hypam fixer and washed in running water for a few minutes. All the solutions were made up according to the manufacturers instructions.

2.21. Protein Determination.

The method of Bearden, (1978) was employed to estimate protein content. Bearden reagent was prepared as follows. Coomassie Brilliant Blue G-250 was dissolved in 85% (w/v) phosphoric acid at a concentration of 1 mg/ml and stirred for 1 hour. This was diluted 5 fold with d.H₂O and filtered through Whatman filter paper to remove undissolved solids. The reagent was stored at room temperature in the dark. Assays contained 0.5 ml Bearden reagent plus 0.5 ml of a suitably diluted protein sample. Absorbance measurements were made at 595 nm using a Pye Unicam SP8-100 ultraviolet spectrophotometer (Cambridge U.K.). The amount of protein in the sample was determined by comparison to a calibration curve constructed using BSA as a standard.

2.22. Protein Western Blotting.

Proteins separated by SDS-PAGE were transferred to nitrocellulose (pore size 0.2 μ m, Schleicher and Schull, Dassel, FRG), using a Bio-Rad Transblot cell (Bio-Rad, Watford, Herts, U.K.). The gel and nitrocellulose were equilibrated separately for at least 30 min in 200 ml of transfer buffer containing 25 mM Tris, 192 mM glycine, pH 8.3, 20% (v/v) methanol. The equilibrated gel was then overlaid on the nitrocellulose, air bubbles were removed and the gel and nitrocellulose were then sandwiched between two pieces of Whatman 3MM filter paper which had

been pre-soaked in transfer buffer. This sandwich was then placed in between 2 sponges and 2 plastic grids and then inserted into the transfer cell containing transfer buffer. The proteins from the gel were then transferred to the nitrocellulose, with the nitrocellulose on the anode side of the gel, at 30 Volts, 0.1 mA at 20⁰C, or 200 mA, constant current when using the mini-blot system, (TE series transphor electrophoresis unit. Hoeffer Scientific Instruments, Biotech, Luton, Bedfordshire, U.K..)

2.23. Visualisation of Proteins Blotted onto Nitrocellulose.

Nitrocellulose blots were stained for less than a minute in 0.1% (w/v) Coomassie Brilliant Blue R, 50% (v/v) methanol and 10% (v/v) acetic acid and then destained in 50% (v/v) methanol and 10% (v/v) acetic acid until bands were visible. Background staining was removed by overnight washing in storage solution containing 5% (v/v) methanol, 7% (v/v) acetic acid. Blots were allowed to air dry.

2.24. Binding of [³H] Verapamil to Proteins Immobilised on Nitrocellulose.

The following procedures were carried out at 20⁰C.

2.24.1. Method 1.

The nitrocellulose blots were washed in 2 x 200 ml of incubation buffer (50 mM Tris, 50 mM Mops, pH 7.5 with HCl), plus 0.05% Tween 20 (to site saturate the blot), for 20 min. The blots were then placed in plastic bags with 10 ml of incubation buffer containing 2.5 nM [³H] verapamil. The bags were sealed and the blots left to incubate for at least 2 hours with agitation. The blots were then washed for 1 hour in 500 ml of incubation buffer. The blots were allowed to dry in a fume cupboard and then sprayed evenly with 3 light coats of EN³HANCE (New England Nuclear, Stevenage, Hertfordshire, U.K.). The blots were allowed to dry and then prepared for autoradiography. After covering the blots with cling film, they were placed face down on a sheet of Hyperfilm, (Amersham, Bucks, U.K.), which had been pre-flashed at a height of 15-20 cm using a flash gun covered by an Ilford S902 filter covered with Whatman No.1 filter paper to diffuse the light (Laskey, 1980). The blot, plus film, were then placed in a cassette at -70°C and developed after 3 weeks using a Gevomatic 60 autoradiograph developer, (Agfa-Geveart, W.Germany).

2.24.2. Method 2.

The nitrocellulose blot was washed in incubation buffer containing 10⁻⁵ M nifedipine for 2 hours in a foil covered container. This was performed in order to site

saturate the blot. Darkness was required as nifedipine is light sensitive. The blot was then washed rapidly in several changes of incubation buffer, to remove any excess nifedipine, and then transferred to a plastic bag containing 10 ml of incubation buffer containing 2.5 nM [^3H] verapamil. After incubation of 2 hours the blot was washed twice for 30 min in 250 ml of incubation buffer. The blot was dried and prepared for autoradiography as described above.

2.24.3. Method 3.

The western blotting procedure was modified in the following ways:

The gel was equilibrated for 30 min in transfer buffer containing 0.1% (w/v) CHAPS, (in order to remove SDS in the gel and renature the proteins). The gel was then laid in between 2 pieces of nylon membrane, (Amersham, Bucks, U.K.), which had been pre-soaked in transfer buffer also, and the sandwich assembled as described previously. The proteins were then transferred from the gel to the nylon membrane at 200 mA, constant current, for 2 hours at 20⁰C, using the mini-blot system. After transfer, the blots were treated as described in method 2, except that the incubation buffer contained 0.1% CHAPS throughout.

2.25. Binding of Con-A to Proteins Immobilised on Nitrocellulose.

Nitrocellulose blots were incubated for 30 min in 3% BSA or 0.05% Tween 20, in Tris buffered saline, (TBS), containing 20 mM Tris, 0.5 M NaCl, pH 7.5 with H_2SO_4 , in order to site saturate the blot. The nitrocellulose was then washed rapidly in several changes of TBS, and then incubated for 1 hour in a plastic bag containing 10 ml TBS, 100 μl Con-A peroxidase (stock batch L4010), 1 mM CaCl_2 and 1 mM MnCl_2 . The blot was then washed for 10 min in 2 changes of TBS, (including 1 mM CaCl_2 and MnCl_2), and developed with Horse Radish Peroxidase (HRP) substrate, (Bio-Rad, Watford, Herts, U.K.). HRP was prepared as follows: At 20°C , 60 mg of HRP reagent was mixed with 20 ml of ice-cold pure methanol. This was added to 100 ml of TBS, (including 1 mM CaCl_2 and MnCl_2), plus 60 μl of ice-cold 30% hydrogen peroxide. After several minutes purple bands developed and the blot was then placed in a large volume of distilled H_2O to stop the development reaction. The blot was kept in darkness to prevent fading of the bands.

2.26. Binding of $^{45}\text{Ca}^{2+}$ to Proteins Immobilised on Nitrocellulose.

The nitrocellulose blot was equilibrated in 4 x 200 ml washes of 60 mM KCl, 5 mM MgCl_2 , 10 mM imidazole, pH 6.8 with HCl. The blot was then incubated in 200 ml of

the above buffer plus 100 μ l of $^{45}\text{Ca}^{2+}$, (approximately 1 μM), for 10 min. After washing the blot in 500 ml of distilled H_2O for 5 min, the blot was left to air dry, then exposed to Hyperfilm for 2-3 weeks in a cassette at -70°C .

2.27. Photoaffinity Labelling of the Phenylalkylamine Binding Protein from Maize Coleoptile Membranes.

2.27.1. Method 1.

100-200 μ l of crude maize coleoptile membrane, (0.5-1.0 mg protein), solubilised maize coleoptile membrane, (0.5 mg protein), in binding buffer (50 mM Tris, 50 mM Mops, pH7.5 with HCl) or DEAE and Sephacryl S400 purified fractions, (0.1-0.5 mg protein), in column buffer, were incubated with a final concentration of 18 nM [^3H] LU 49888 (total binding) or 18 nM [^3H] LU 49888 in the presence of 10^{-4}M (final concentration) of unlabelled D888 (non-specific binding). After incubation for 2 hours in the dark at room temperature, the suspensions were placed in disposable microtitre plates and irradiated at a distance of 4 cm with a Blak-Ray long wave ultraviolet lamp, (model B-100A. Ultraviolet products, San Gabriel, California, U.S.A.), for 10 min on ice. Samples for gels were then prepared as described previously and subjected to electrophoresis on 10% polyacrylamide gels. Molecular weight markers (see section 2.18.5.) were separated by SDS-PAGE at the same time.

Following electrophoresis, gel slabs were either:

1. Fixed and stained with Coomassie blue and then dried down onto Whatman filter paper under vacuum and cut into 2 mm slices. The slices were solubilised for 48 hours in Protosol, (New England Nuclear, Stevenage, Herts.), or 0.1 M NaOH and the radioactivity in each slice determined in aqueous scintillation fluid using a scintillation counter.
2. Fixed and stained with Coomassie blue and then incubated in EN³HANCE, (New England Nuclear, Stevenage, Hertfordshire, U.K.), for 1 hour, followed by incubation for 30 min in distilled H₂O. The gel slab was then dried down under vacuum and exposed to pre-flashed Hyperfilm-³H for 2-3 weeks at -70°C, as described previously.

2.27.2. Method 2.

100 µl of crude membrane resuspension was incubated in binding buffer, (50 mM Tris, 50 mM Mops, pH 7.5 with HCl), with a final concentration of 18 nM [³H] LU 49888, (total binding), or 18 nM [³H] LU 49888 plus 10⁻⁴ (final concentration) of unlabelled D888, (non-specific binding), in a total volume of 1 ml. All assays were carried out in duplicate. The suspension was incubated for 2 hours in the dark at 20°C. After incubation, 3 ml of ice-cold wash buffer, (10% PEG 8000, 50 mM Tris, pH 7.5 with HCl), was added to the suspension, which was then filtered through a Whatman GF/C filter under vacuum. The filter was then washed once with 3 ml of ice-cold

wash buffer and then placed on ice and irradiated for 10 min with ultraviolet light at a distance of 4 cm. One set of filters were placed directly into scintillation vials with 5 ml of aqueous scintillant and the radioactivity retained on the filter determined using a scintillation counter. The remaining set of filters were placed in eppendorfs with 500 μ l of sample buffer (see section 2.18.3.) and boiled for 20 min in a hot block, (Dri block, Cambridge, U.K.). The eppendorfs were allowed to cool and the filter removed. Insoluble material was pelleted by centrifugation at 11,600 g for 5 min. A portion of the remaining soluble sample was then applied to a 10% SDS Polyacrylamide gel for electrophoresis. Molecular weight markers were also separated by SDS-PAGE at the same time. After electrophoresis the gel was stained, dried down and cut into 2 mm slices which were solubilised in 0.1 M NaOH and the radioactivity in each slice determined in aqueous scintillation fluid using a scintillation counter.

2.28. Protein Kinase Assays.

2.28.1. Buffers.

The following buffers were used in the protein kinase assays.

Buffer A. 22.2 mM MgCl_2 , 111 mM HEPES, 0.89 mM EGTA, 1.09 mM CaCl_2 . The buffer was made at 2x concentration so that a free concentration of 100 μM Ca^{2+} was achieved when the buffer was diluted.

Buffer B. As for buffer A, minus CaCl_2 .

Buffer C. 6 μM Bovine calmodulin in buffer A. After dilution a concentration of 3 μM calmodulin was achieved. TCA/Na pyrophosphate/EDTA solution. 20 mM NaPP, 10 mM EDTA, 10% TCA.

2.28.2. The cellulose disc assay for crude membrane fractions.

15 μl of crude membrane resuspension was mixed with an equal volume of either buffer A, B or C. [γ - ^{32}P] ATP was added (approx. 8 nM), aliquots removed after 1 min and spotted onto Whatman cellulose discs which had been pre-treated with 0.1 ml TCA/NaPP/EDTA solution. The filters were then washed overnight in a large volume of TCA/NaPP/EDTA solution. After removing the TCA/NaPP/EDTA solution, the discs were soaked in 25% (w/v) TCA for 20 min and then transferred to boiling TCA/NaPP/EDTA for 15 min. After cooling for 30 min the discs were washed in acetone containing 2% (v/v) 1 M HCl and air dried. Incorporated phosphate was estimated by Cerenkov counting of each disc in 5 ml d. H_2O . Blanks containing no protein, were also assayed.

2.28.3. The phosphocellulose strip assay for solubilised membrane and column fractions.

The solubilised membrane fractions or column fractions were mixed with an equal volume of either buffer A, B or C. [γ - ^{32}P] ATP at a final concentration of

either 8 nM or 1 μ M was added and aliquots were spotted onto 1 x 2 cm Whatman P81 phosphocellulose strips at the times indicated in the figure legends. The strips were then immediately placed into 75 mM phosphoric acid at 10 ml/strip. Blanks were added last since desorption of ATP is time dependent. The strips were washed 3 times in phosphoric acid and then allowed to air dry. Incorporated phosphate was estimated by Cerenkov counting of each strip in 5 ml of d. H_2O .

Samples for gel electrophoresis were prepared as described previously. After the gels had been coomassie stained and dried down they were exposed to Hyperfilm for upto 2 weeks at room temperature and then developed in a Gevomatic 60 autoradiograph developer.

CHAPTER 3.

THE CHARACTERISATION OF [^3H] VERAPAMIL BINDING IN CRUDE MEMBRANE PREPARATIONS FROM MAIZE COLEOPTILES.

3.1. Introduction.

Radiolabelled drugs such as nifedipine, PN200-110 (a DHP) and verapamil have been used to identify and subsequently purify the voltage-dependent calcium channel from skeletal muscle T-tubule membranes, (Curtis & Catterall 1984; Borsotto *et al.*, 1985). Various pieces of evidence outlined in the introduction to this thesis, suggest that calcium channels exist in plant cell membranes (Andrejauskas *et al.*, 1985; Graziana *et al.*, 1987). The first aim of this project was to detect calcium channel proteins in maize coleoptile tissue using [^3H] verapamil and a related phenylalkylamine, [^3H] desmethoxyverapamil (D888).

The results presented in this chapter are:-

1. Development of the verapamil binding assay and the subsequent detection of phenylalkylamine binding proteins in plant membranes using the assay.
2. Determination of the specific binding constants of verapamil and D888 and estimates of binding protein/channel density.
3. Estimation of the binding specificity of a variety of calcium channel antagonists.
4. Localisation of binding sites according to tissue type and membrane type.

3.2. Principles of the binding assay.

The principles behind the binding assay are presented here to define the terms used in this and the following results sections.

Total binding refers to the amount of labelled calcium channel antagonist that binds to the membrane fractions. This is determined by adding labelled antagonist only to the membrane fractions. Under these circumstances the labelled antagonist binds to both high and low affinity binding sites. To distinguish between these, unlabelled antagonist is added to displace the labelled drug from any high affinity binding sites. The amount displaced depends on the concentration of the unlabelled antagonist and is known as specific binding, saturable binding or exchangeable binding. The term specific binding is used in this thesis. It should be noted that this term is not strictly accurate as the assay does not indicate anything about the specificity of the binding. However the term does distinguish between binding that is high affinity binding and weaker types of binding, whether saturable or not. Binding activity remaining at high concentrations of unlabelled drug is attributed to non-specific, non-saturable or non-exchangeable binding. This comprises of low affinity binding and partitioning of labelled antagonist into membrane vesicles and spaces between vesicles. The term non-specific binding is used in this thesis.

3.3. Development of the [^3H] verapamil binding assay.

3.3.1. *The centrifugation assay.*

Initial attempts to detect [^3H] verapamil and [^3H] D888 binding in crude membrane preparations from maize coleoptiles, were made using a centrifugation assay (Ray *et al.*, 1977; Venis, 1985). A brief description of the assay is presented here. Labelled antagonist was allowed to bind to crude membrane fractions in the presence or absence of unlabelled antagonist. The membranes were then separated from any unbound label by centrifugation and the resulting pellet rinsed to remove remaining liquid and excess label. The amount of bound label was determined by scintillation counting of the resuspended pellet.

Fig.3.1. shows a typical displacement curve for [^3H] verapamil binding in crude membrane preparations from maize coleoptiles determined using the centrifugation assay. The graph indicates that at all the concentrations tested, the unlabelled verapamil was unable to significantly displace the labelled verapamil. At the highest concentration of unlabelled verapamil (100 μM), non-specific binding comprised 70% of the total binding. Due to these high levels of non-specific binding, probably caused by the trapping of labelled antagonist within membrane vesicles at the pelleting stage of the assay, the centrifugation assay was not adopted as the standard method to detect [^3H] verapamil binding.

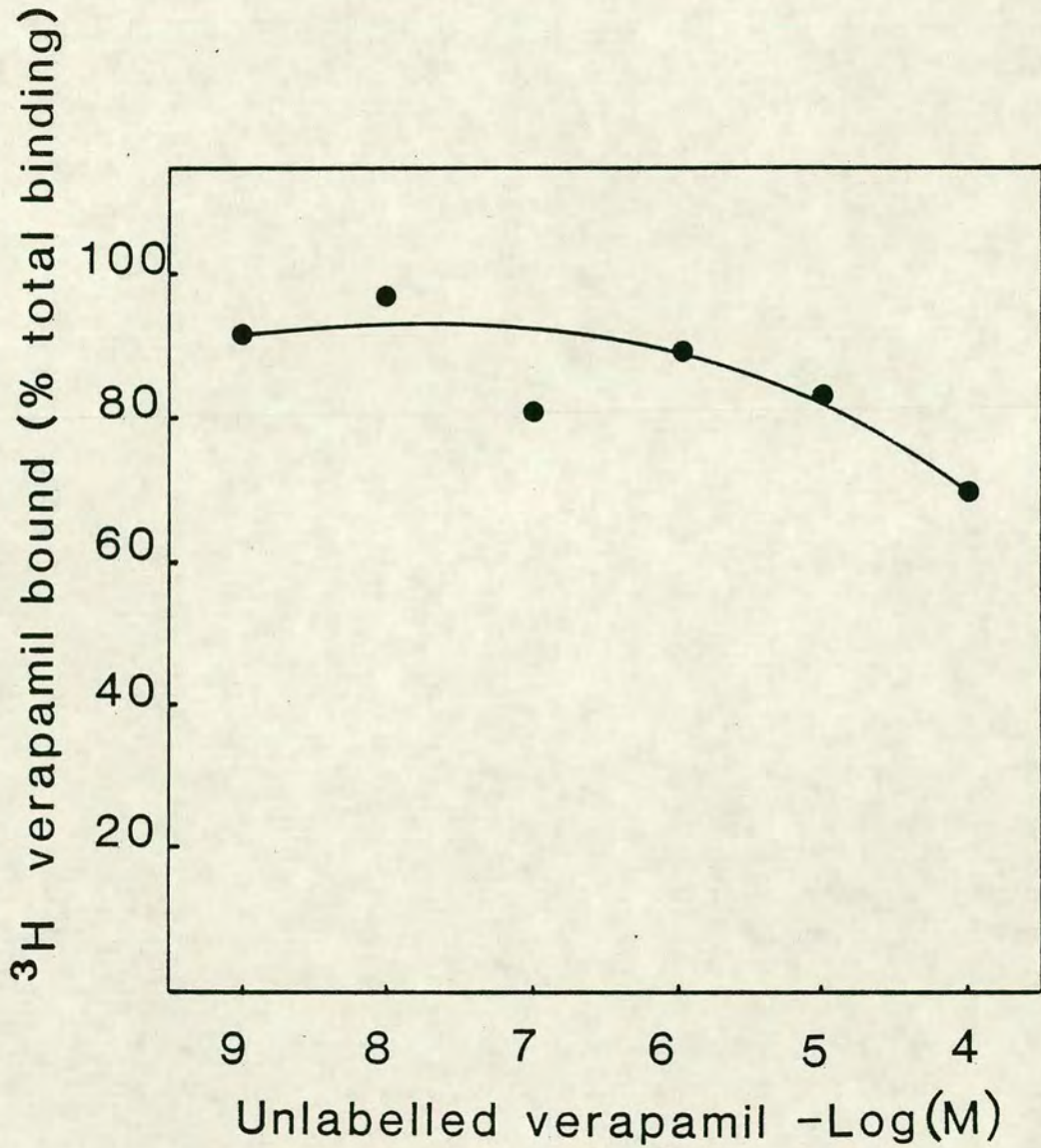


Fig.3.1. Displacement of [^3H] verapamil by unlabelled verapamil measured using the centrifugation assay.

[^3H] verapamil was added to the crude maize coleoptile membrane fraction (0.4 mg/ml assay), to give a final concentration of 0.3 nM. Unlabelled verapamil was then added at the concentrations indicated. After incubation for 120 min at 20°C the mixtures were pelleted by centrifugation. The pellets were resuspended and the amount of radioactive label bound to the membrane determined by liquid scintillation counting. Total [^3H] verapamil binding i.e. 100% was 2500 c.p.m.

3.3.2. The filter assay.

The filter assay, modified from Andrejauskas *et al.* (1985), was then used to assay for [^3H] verapamil binding to crude membrane fractions. In this method labelled antagonist was allowed to bind to crude membrane fractions in the presence or absence of unlabelled antagonist. The membranes were separated from any unbound label by filtration and the filters washed to remove any excess label. The label bound to the membrane was determined by liquid scintillation counting of the filters. This method gave a better distinction between specific and non-specific binding. This was probably because membrane vesicles which may trap any free label available, so increasing non-specific binding, do not form on the filters. Washing of the filters also appears to be more successful in removing any free label present, compared to washing of the pellets in the centrifugation technique.

Having achieved lower levels of non-specific binding with the filter method, the filter assay was subsequently chosen as the standard method for assaying [^3H] verapamil and [^3H] D888 binding in membrane preparations and used in all further experiments. Fig.3.3. shows a typical displacement curve for [^3H] verapamil binding in crude membrane preparations using the filter assay method.

3.4. The kinetics of [^3H] verapamil binding to maize coleoptile membranes.

To ensure that [^3H] verapamil binding had reached equilibrium, the kinetics of drug binding were investigated using the standard filter assay.

Fig.3.2. shows the binding of [^3H] verapamil at 20°C , pH 7.5 to crude membrane fractions and its displacement by 100 μM unlabelled verapamil. The graph indicates that binding of [^3H] verapamil increases over time, with binding equilibrium being reached after 90 min. When 100 μM unlabelled verapamil was added at the plateau phase, dissociation of bound verapamil occurred and was largely complete after a further 120 min.

As a consequence of these results, incubations of membrane fractions with [^3H] verapamil were conducted for 2 hours.

3.5. [^3H] verapamil and [^3H] D888 binding to maize coleoptile membranes.

Binding curves for [^3H] verapamil and [^3H] D888 were constructed using the standard binding assay.

Fig.3.3. shows the displacement of [^3H] verapamil by increasing concentrations of unlabelled verapamil. The graph indicates that the amount of displacement increases with increasing concentration of unlabelled drug. 100 μM unlabelled verapamil was able to displace the [^3H] verapamil by 96%. The remaining 4% was considered to be non-specific binding.

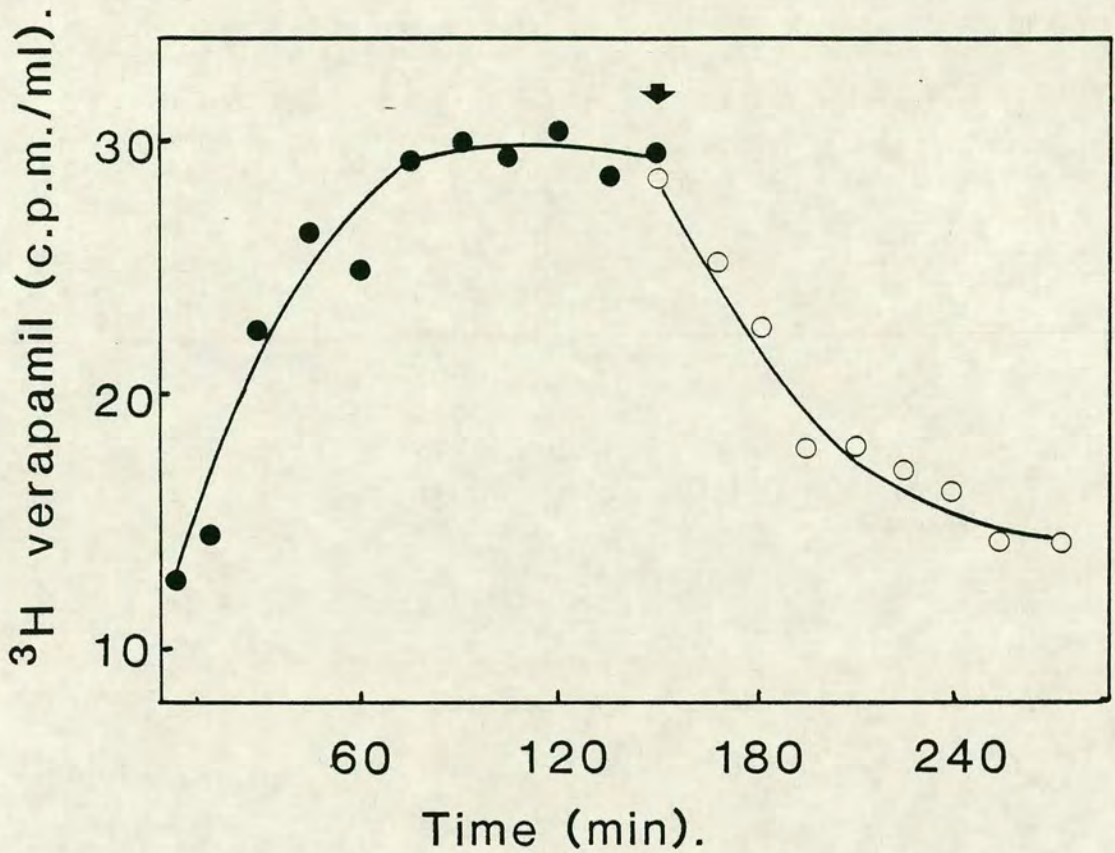


Fig.3.2. The kinetics of [^3H] verapamil binding to maize coleoptile membrane fractions.

Binding was initiated (●), by the addition of [^3H] verapamil (final concentration. 1.12 nM) to the incubation mixture containing membrane protein (0.38 mg/500 μl assay). At the times indicated, 250 μl samples were withdrawn and the amount of [^3H] verapamil binding determined by the filter assay. Dissociation of [^3H] verapamil binding (○), was initiated by the addition of 100 μM unlabelled verapamil at the point indicated by \blacktriangledown .

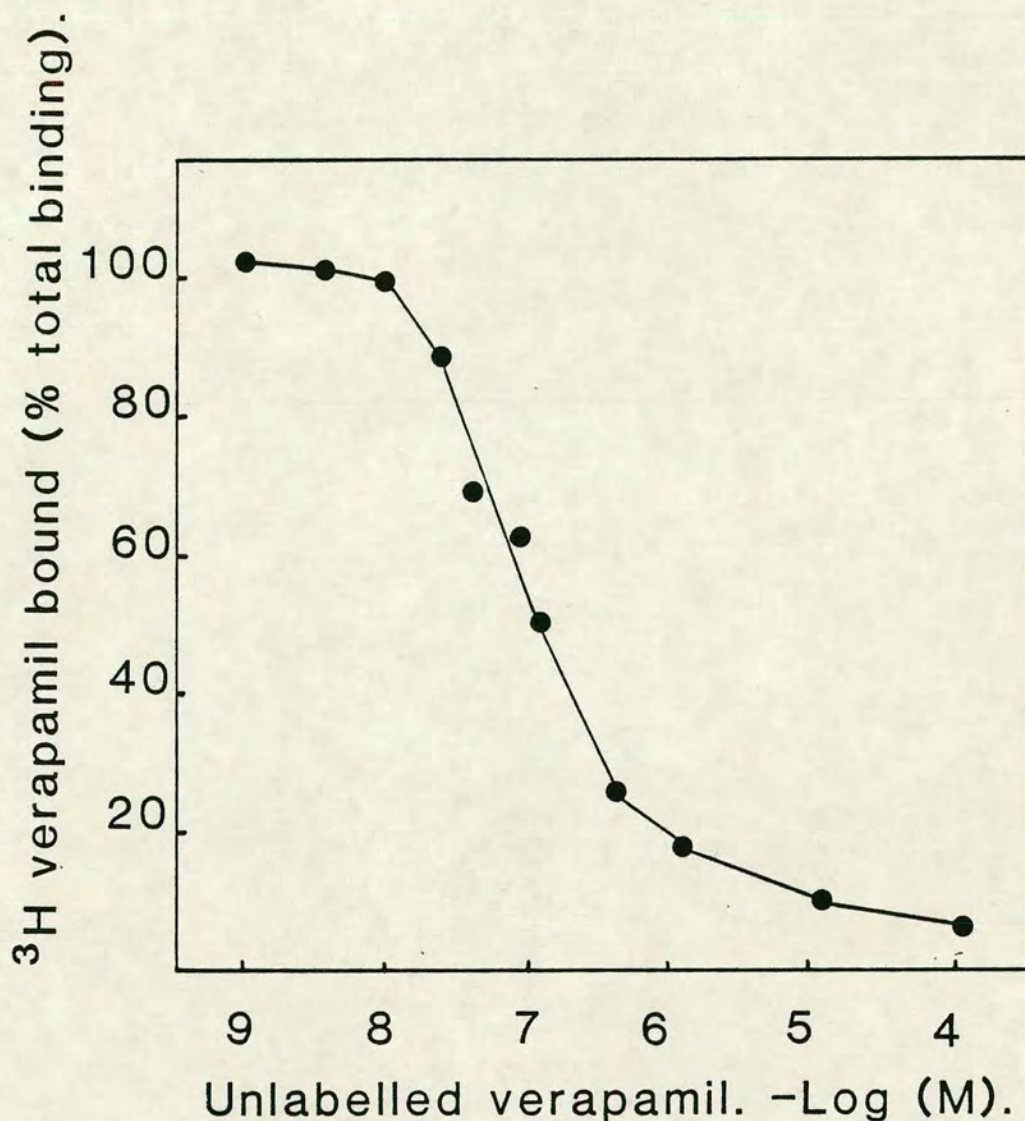


Fig.3.3. Displacement of [³H] verapamil by unlabelled verapamil measured using the filter assay.

[³H] verapamil was added to the crude maize coleoptile membrane fraction (0.5 mg/500 μ l assay) to give a final concentration of 2.24 nM. Unlabelled verapamil was added at the concentrations indicated. The radioactivity bound to the membrane was determined using the standard filter assay. Total [³H] verapamil binding was 196 c.p.m.

The displacement of [^3H] D888 by unlabelled D888 is shown in Fig.3.4. The graph indicates that at low concentrations, the unlabelled D888 does not significantly displace the labelled D888. At 100 μM unlabelled D888 the amount of non-specific binding was 50% of the total binding. It is also noticeable that the unlabelled D888 is unable to displace the labelled D888 to the same levels as the unlabelled verapamil displaced the [^3H] verapamil. These results indicate low affinity binding of D888 in maize coleoptile membranes.

The graph also indicates an element of positive cooperativity in the region of the graph where low concentrations of D888 (10^{-8}M) were used to displace [^3H] D888 binding. Positive cooperativity occurs when the binding of one molecule of the drug facilitates the binding of subsequent molecules of the drug. This can happen because of a conformational change in the binding sites caused by the binding of the first molecule, which then alters the K_D of the sites, (they become higher affinity or binding sites become revealed), so allowing more molecules to bind. In this case it would appear that the addition of 10^{-8}M - 10^{-7}M unlabelled D888 facilitates the binding of more labelled D888, possibly by the above mechanism, which may have been induced by the slight rise in total D888 concentration.

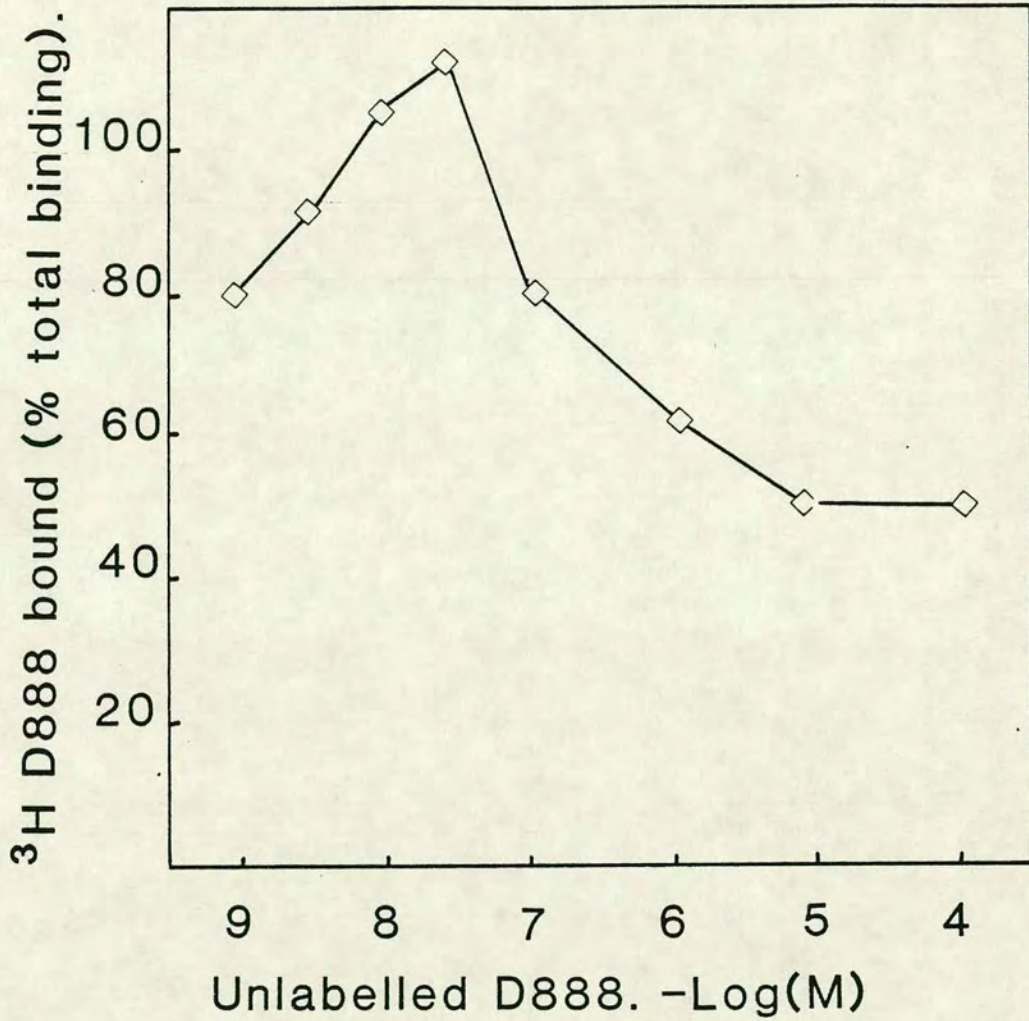


Fig.3.4. Displacement of [³H] D888 by unlabelled D888.

[³H] D888 was added to the crude membrane fraction (0.5 mg/500 μ l assay), to give a final concentration of 2.3 nM. Unlabelled D888 was added at the concentrations indicated. The amount of radioactivity bound to the membrane was determined using the standard filter assay. Total [³H] verapamil binding was 100 c.p.m.

3.6. Specific binding constants of verapamil & D888 and the estimation of binding protein density.

Data from [^3H] verapamil and [^3H] D888 displacement experiments were transformed by the method of Scatchard (1949), to give Scatchard plots. From these, the apparent dissociation constant (K_D) and the maximum number of binding sites (B_{max}) were calculated from the slope of the line of best fit and the intercept of the line on the x axis respectively.

Fig.3.5. shows a typical Scatchard plot of [^3H] verapamil binding to crude membrane preparations from maize coleoptiles. The Scatchard plot is linear which suggests that there is a single class of non-interacting binding sites. The apparent K_D was 72 nM and the B_{max} was 135 pmol/mg protein indicating a fairly high affinity binding site.

A typical Scatchard plot of [^3H] D888 binding to crude maize coleoptile membranes is shown in Fig.3.6. The Scatchard plot appears to be linear, suggesting a single class of D888 binding sites. It is possible that there may be another binding site, but in the absence of computerised line fitting is not detected using the least squares method to construct the line of best fit. Binding of D888 was of lower affinity compared to verapamil binding, with a K_D of 578 nM and a B_{max} of 220 pmol/mg protein.

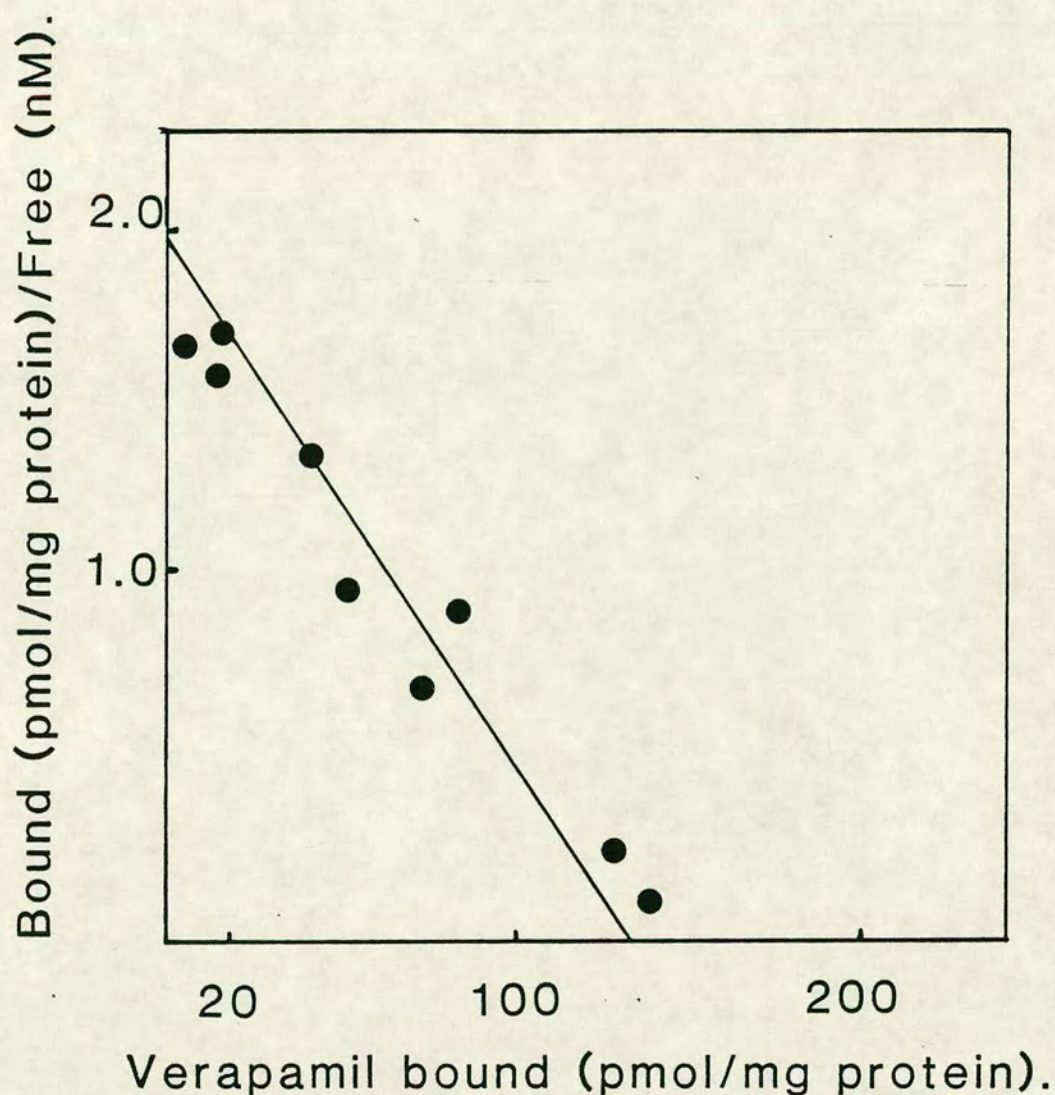


Fig.3.5. Scatchard plot of [^3H] verapamil binding to crude membrane fractions from maize coleoptiles. Data from [^3H] verapamil displacement experiments was transformed to give a Scatchard plot. Least squares regression analysis was used to plot the line of best fit. The apparent K_D was 72 nM and the B_{max} was 135 pmol/mg of protein.

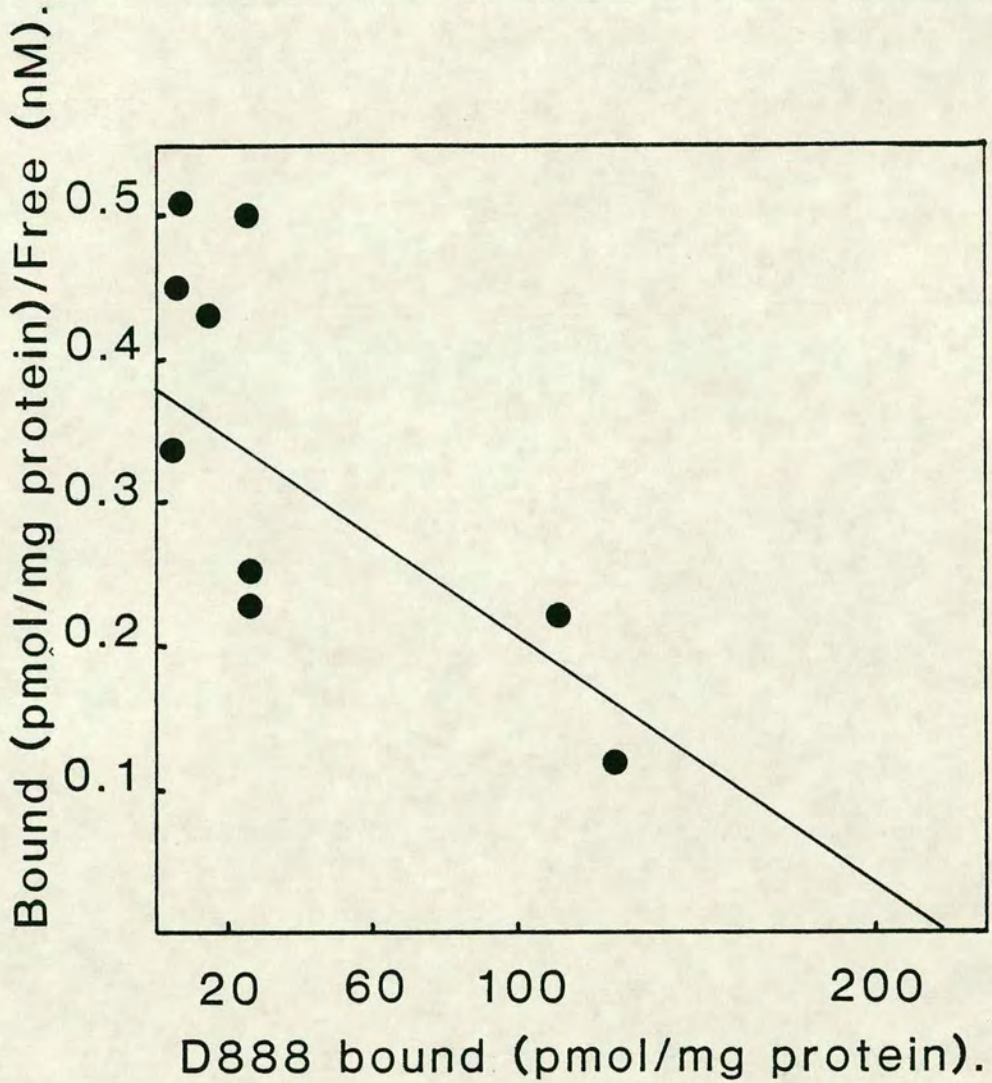


Fig.3.6. Scatchard plot of [³H] D888 binding to crude membrane fractions from maize coleoptiles.

Data from [³H] D888 displacement experiments was transformed to give a Scatchard plot. Least squares regression analysis was used to plot the line of best fit. The apparent K_D was 579 nM and the B_{max} was 220 pmol/mg of protein.

3.7. The displacement of [^3H] verapamil and [^3H] D888 by unlabelled calcium channel antagonists.

In displacement experiments using skeletal muscle microsomes, calcium antagonists from one structural group were found to be able to displace calcium antagonists from another group, but not as well as those from the same series. These results suggested that there are separate binding sites for the different drugs and that these sites may be linked (Glossmann *et al.*, 1982; Triggle *et al.*, 1986). To investigate whether separate calcium antagonist binding sites exist in plant membranes, the displacement of [^3H] verapamil and [^3H] D888 by unlabelled calcium channel antagonists was performed.

3.7.1. [^3H] verapamil displacement.

Fig.3.7. shows the displacement of [^3H] verapamil by unlabelled verapamil, D888, methoxyverapamil (D600) and nifedipine. The graph shows that over the concentration range tested, only verapamil and D600 were able to appreciably displace [^3H] verapamil binding in maize coleoptile membranes, with IC_{50} values of 100 nM and 700 nM respectively. D888 competed poorly for the [^3H] verapamil binding sites and had an IC_{50} value of 50 μM . Nifedipine had little displacement effect even at 10^{-4} M.

3.7.2. [^3H] D888 displacement.

Displacement of [^3H] D888 by unlabelled D888, D600

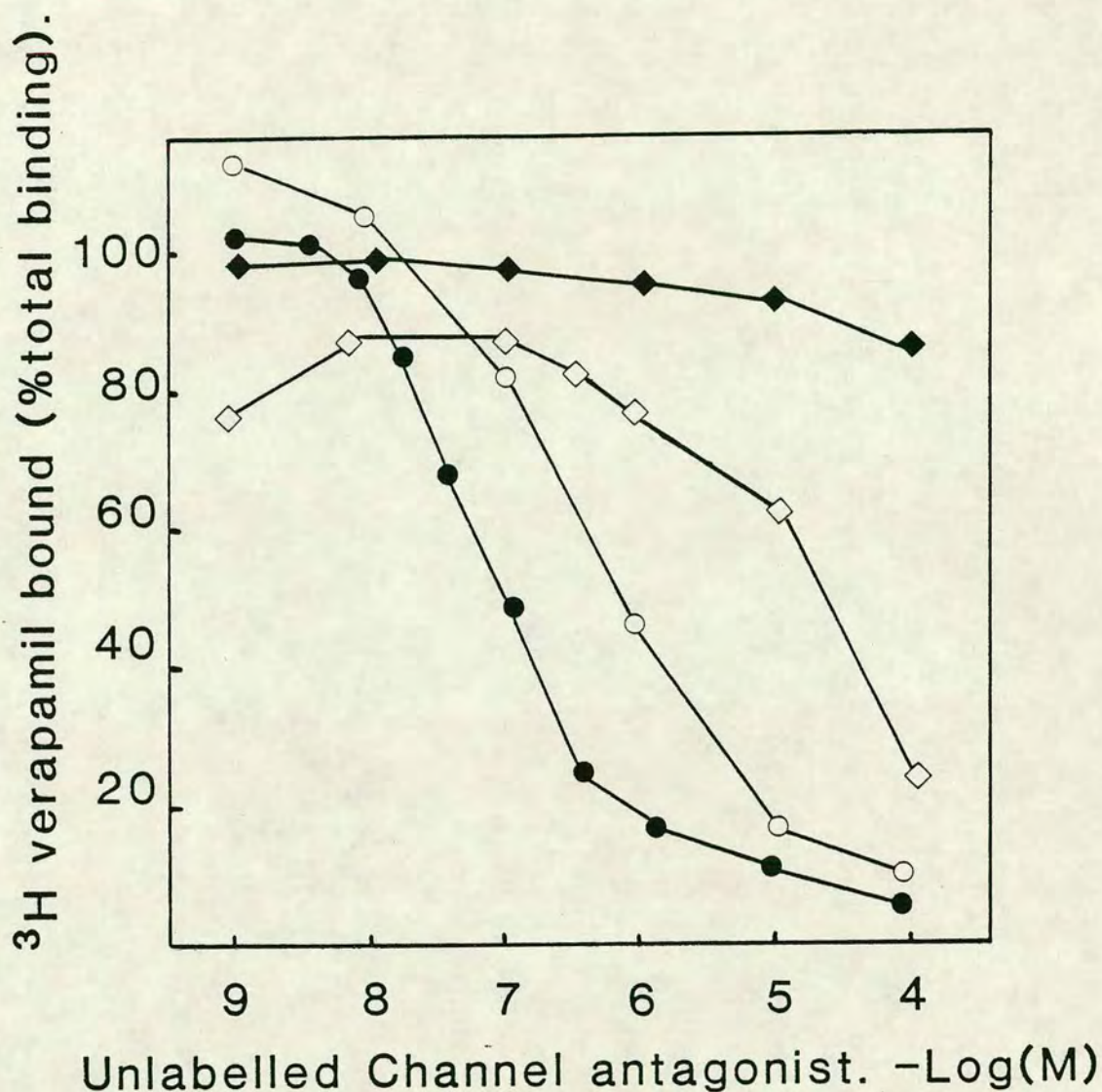


Fig.3.7. Displacement of [^3H] verapamil by unlabelled calcium channel antagonists.

Binding of [^3H] verapamil to crude maize coleoptile membrane fractions was measured using the standard filter assay in the presence of either unlabelled verapamil (●), methoxyverapamil (○), desmethoxyverapamil (◇) or nifedipine (◆) at the concentrations indicated. The value for total [^3H] verapamil binding was 196 c.p.m. (250 f mol). Non-specific binding, determined by the addition of 100 μM unlabelled verapamil, was 4% of the total.

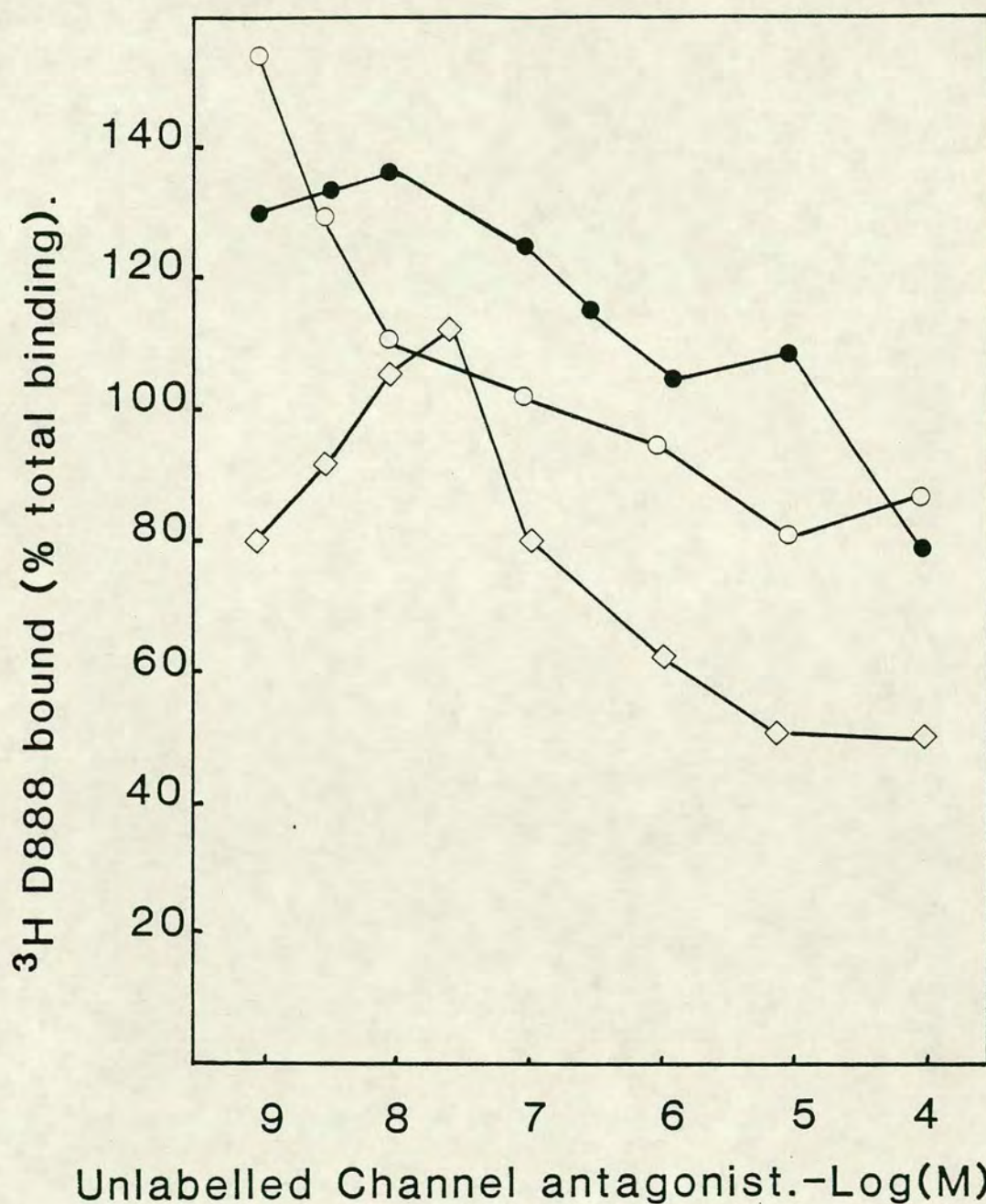


Fig.3.8. Displacement of [³H] D888 by unlabelled calcium channel antagonists.

Binding of [³H] D888 to crude maize coleoptile membrane fractions was measured using the standard filter assay in the presence of either unlabelled verapamil (●), methoxyverapamil (○) or desmethoxyverapamil (◇) at the concentrations indicated. The value for total [³H] D888 binding was 100 c.p.m. Non-specific binding, determined by the addition of 100 μm unlabelled D888 was 50% of the total.

and verapamil is shown in Fig.3.8. In all cases displacement of the labelled antagonist from its binding sites was poor. Unlabelled D888 was able to displace approximately 50% of the labelled D888 at 10^{-4} M. Unlabelled verapamil appeared to have no significant effect up to 10^{-4} M, where it displaced approximately 30% of the [3 H] D888 binding. D600 was able to displace [3 H] D888 over the range of 10^{-7} M - 10^{-4} M unlabelled D600, by approximately 50-70%.

3.8. Localisation of [3 H] verapamil binding sites according to tissue type and membrane type.

3.8.1. *Tissue type.*

[3 H] verapamil binding studies were carried out using maize root and zucchini hypocotyl membranes, as well as maize coleoptile membranes. Figs.3.9 a & b. indicate the binding curve for [3 H] verapamil in maize root tissue and the Scatchard plot derived from this data. For maize roots the K_D was found to be 233 nM, which represents a relatively low affinity binding site compared to that found for maize coleoptiles. The B_{max} was found to be 165 pmol/mg protein (Table 3.1.). This is a comparatively high density of binding sites than found for maize coleoptile tissue.

Figs.3.10 a & b. show the binding curve and associated Scatchard plot for [3 H] verapamil binding in zucchini hypocotyl tissue. The K_D was found to be 114 nM and the B_{max} 96 pmol/mg protein (Table 3.1.). This data

Fig.3.9.a. The displacement of [^3H] verapamil by unlabelled verapamil in crude membrane fractions from maize roots.

[^3H] verapamil was added to crude maize root membrane fractions (0.4 mg/500 μl assay) to give a final concentration of 2.24 nM. Unlabelled verapamil was added at the concentrations indicated. The amount of [^3H] verapamil bound was measured using the standard filter assay. Total [^3H] verapamil binding was 253 c.p.m.

b. Scatchard plot of [^3H] verapamil binding to crude membrane fractions from maize roots.

Data from Fig.3.9.a. was transformed to give a Scatchard plot. Least squares regression analysis was used to plot the line of best fit. The apparent K_D was 233 nM and the B_{max} was 165 pmol/mg of protein.

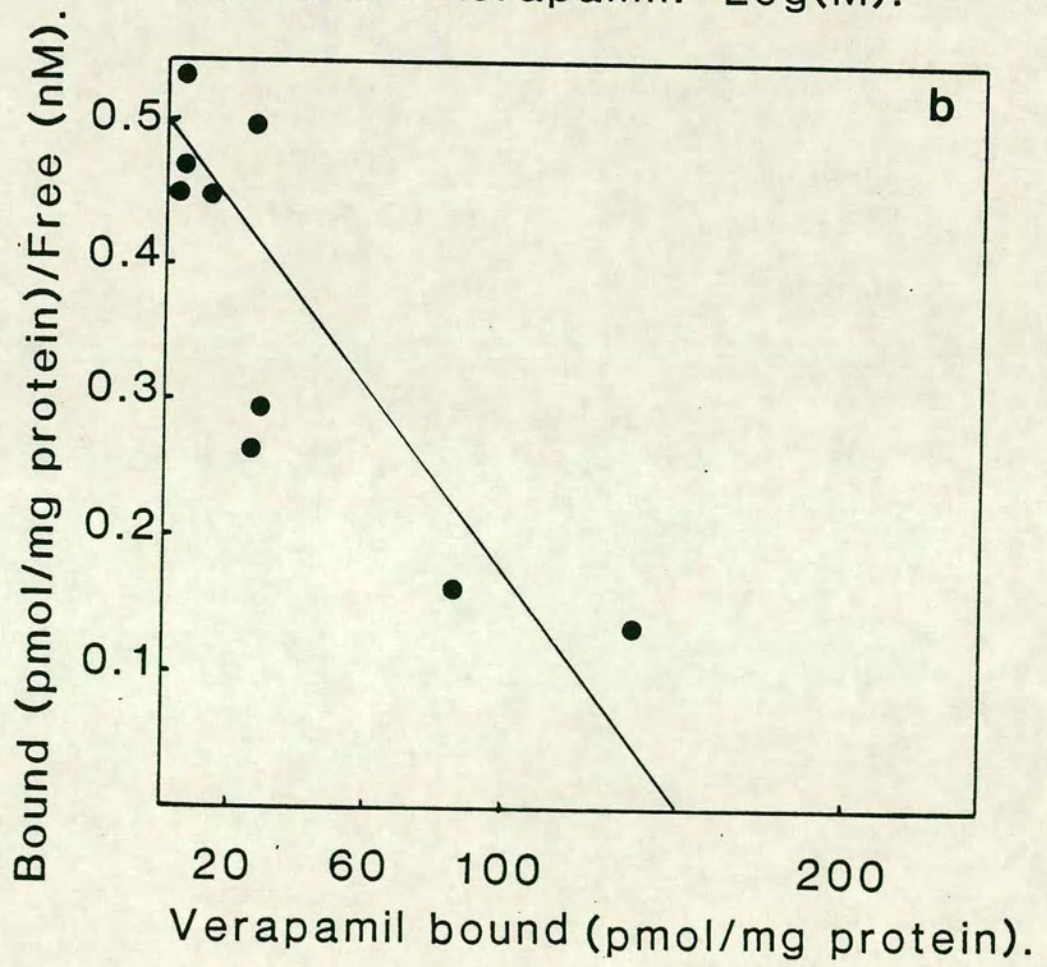
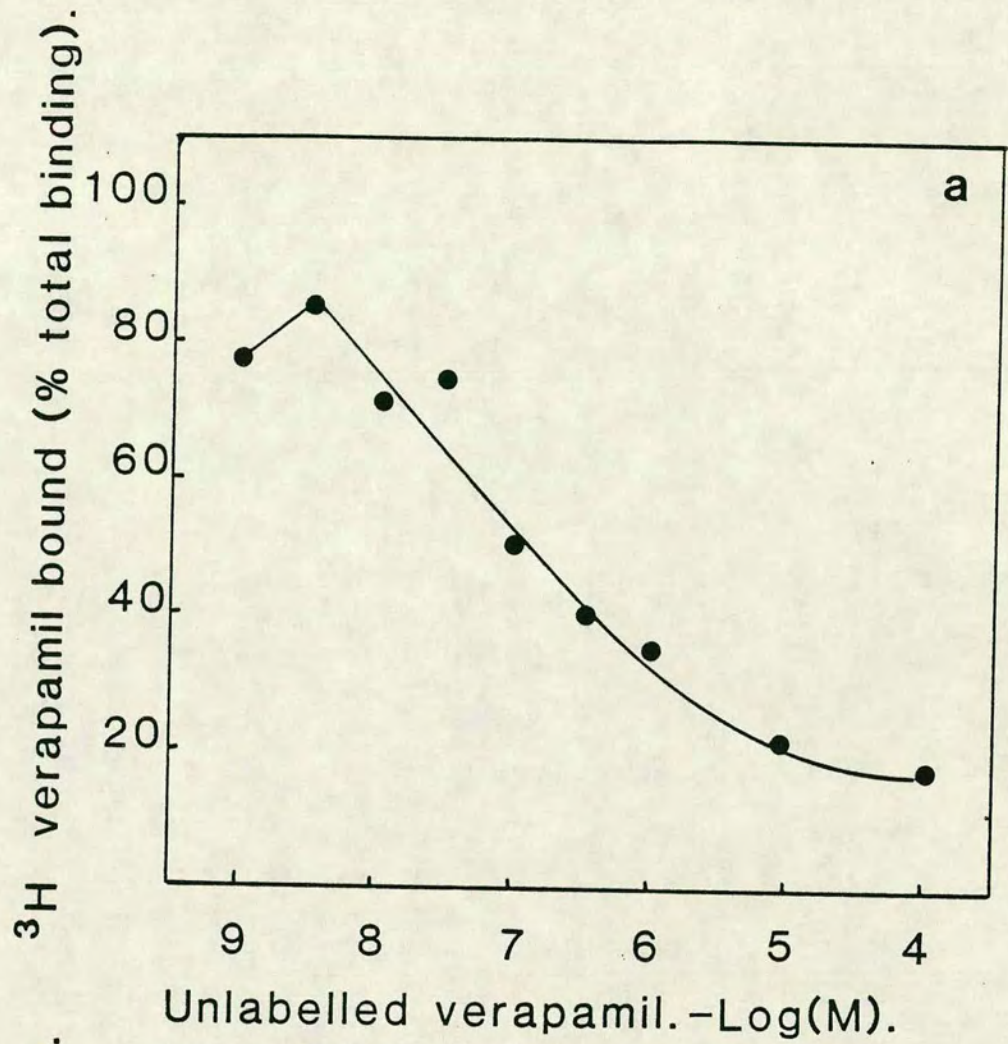
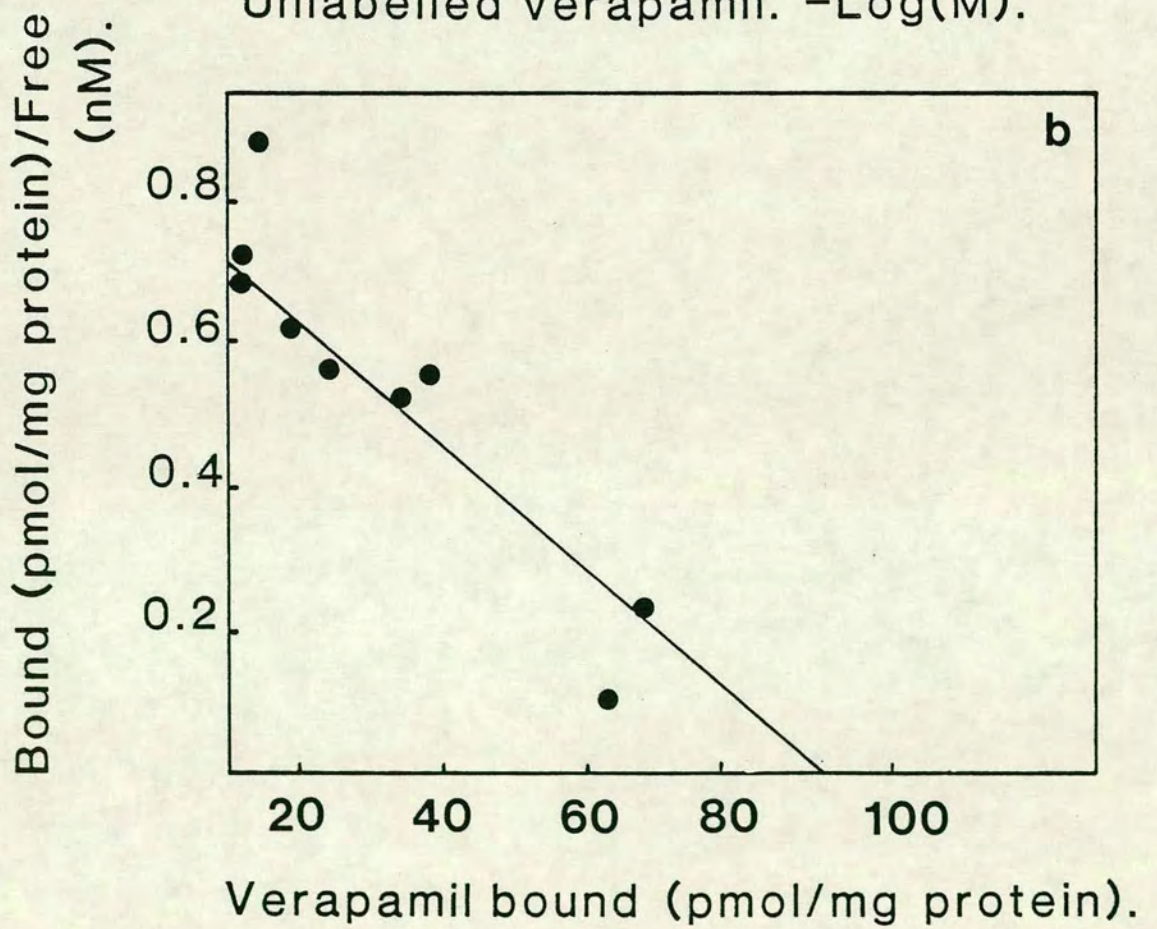
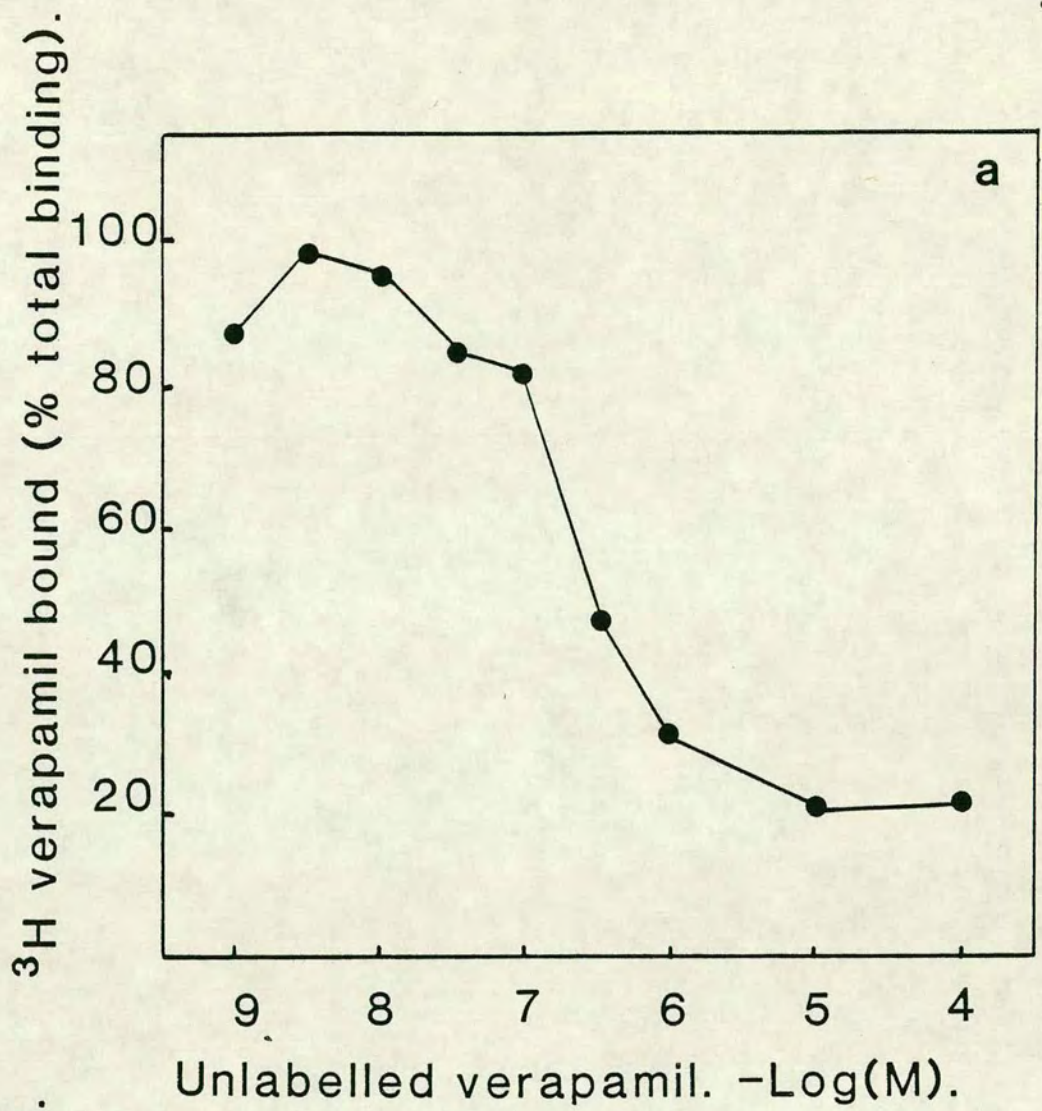


Fig.3.10.a. The displacement of [^3H] verapamil by
unlabelled verapamil in crude membrane
fractions from zucchini hypocotyls.

[^3H] verapamil was added to crude membrane fractions from zucchini hypocotyls (0.35 mg/500 μl assay) to give a final concentration of 2.24 nM. Unlabelled verapamil was added at the concentrations indicated. The amount of [^3H] verapamil bound to the membrane was determined using the standard filter assay. Total [^3H] verapamil binding was 189 c.p.m.

b. Scatchard plot of [^3H] verapamil binding to
crude membrane fractions from zucchini
hypocotyls.

Data from Fig.3.10.a. was transformed to give a Scatchard plot. Least squares regression analysis was used to plot the line of best fit. The apparent K_D was 114 nM and the B_{max} was 96 pmol/mg of protein.



<u>Tissue Type.</u>	<u>Dissociation Constant. K_D [nM].</u>	<u>Maximum No. Binding sites. pmol/mg protein.</u>	<u>Reference.</u>
<u>Maize coleoptiles.</u>	72	135	*
<u>Maize roots.</u>	233	165	*
<u>Zucchini hypocotyls.</u>	114	96	*
<u>Zucchini hypocotyls.</u>	102	60	Andrejauskas et al., 1985.
<u>Rabbit Skeletal muscle.</u>	27	50	Galizzi et al., 1984.
<u>Guinea-Pig Skeletal muscle.</u>	45	37 (fmol)	Goll et al., 1984.
<u>Rat cerebral cortex.</u>	93	435	Reynolds et al., 1983.

Table 3.1. [³H] verapamil binding in various plant and animal tissues.

* denotes data obtained by the author.

is comparable to that found by Andrejauskas *et al.* (1985), who reported a K_D of 102 nM and a B_{max} of 60 pmol/mg protein for zucchini hypocotyl membrane fractions.

3.8.2. Membrane type.

To investigate calcium channel localisation according to membrane type, preliminary studies were made utilising the phase partitioning of total membrane fractions according to the method of Yoshida *et al.* (1983). During partitioning the plasma membrane preferentially moves into the upper polyethyleneglycol phase due surface properties and charge. It has been reported that only "right-side-out" sealed plasma membrane vesicles partition into the upper phase (Larsson *et al.*, 1984).

Plasma membrane enriched upper phase and residual membrane lower phase (containing ER, tonoplast and some plasma membrane), were prepared and assayed for [3H] verapamil binding using the standard binding assay. The results indicated the general distribution of the calcium channel proteins according to membrane type. Table 3.2. summarises [3H] verapamil binding to the plasma membrane enriched fraction and residual membrane fraction from maize tissues.

The results indicate that for all the tissues examined there is increased verapamil binding in the plasma membrane enriched upper phase compared to the

<u>Tissue</u>	<u>Verapamil bound (nmols/mg protein).</u>	
	<u>Upper phase.</u>	<u>Lower phase.</u>
<u>Coleoptiles incl. leaf rolls.</u>	15.7	1.25
<u>Coleoptiles excl. leaf rolls.</u>	4.4	2.3
<u>Leaf rolls.</u>	15.2	1.0
<u>Roots</u>	4.9	2.5

Table 3.2. [^3H] verapamil binding to the plasma membrane enriched upper phase and the residual membrane lower phase, from various maize tissues.

Plasma membrane was separated from the other membrane types using the two-phase method developed by Yoshida et al., 1983. Each phase was assayed for [^3H] verapamil binding using the standard filter assay.

residual membrane lower phase. This suggests that there are more calcium channels in the plasma membrane compared to the other membrane types.

The amount of verapamil bound by the plasma membrane enriched fraction also varies according to the tissue source the plasma membrane was derived from. Table 3.2. indicates that plasma membrane from leaf rolls only and from coleoptiles plus leaf roll, bound the most [^3H] verapamil. Coleoptiles alone only bound a small amount of verapamil, suggesting that it is the leaf roll which actually binds the most [^3H] verapamil. This suggests that the plasma membrane from the leaf has more calcium channels than the coleoptile plasma membrane. Plasma membrane from root tissue also bound less verapamil than plasma membrane from leaf rolls.

3.9. Discussion.

Using the standard filter method to assay for [^3H] verapamil binding, crude maize coleoptile membranes have been shown to bind verapamil. This binding is saturable and reversible. [^3H] verapamil binding takes 2 hours to reach equilibrium at 20°C , pH 7.5 and on addition of 100 μM unlabelled verapamil the [^3H] verapamil dissociates and binding levels off after a further 2 hours. These data can be compared to those obtained for verapamil binding to skeletal muscle microsomes and zucchini hypocotyl membranes, both of which also took 2 hours to

reach equilibrium (Goll *et al.*, 1984a; Andrejauskas *et al.*, 1985).

Scatchard analysis of [^3H] verapamil binding in crude maize coleoptile membrane fractions resulted in a linear plot, suggesting a single class of non-interacting binding sites. A K_D of 72 nM and B_{max} of 135 pmol/mg protein was obtained. The dissociation constant is similar to that obtained for [^3H] verapamil binding in zucchini (K_D = 102 nM, B_{max} = 60 pmol/mg protein), but is of lower affinity than that obtained for [^3H] verapamil binding in skeletal muscle where the K_D was 27 nM and the B_{max} was 50 pmols/mg protein (Andrejauskas *et al.*, 1985; Galizzi *et al.*, 1984a).

Scatchard plots of [^3H] D888 binding indicated an apparent K_D of 578 nM and a B_{max} of 220 pmol/mg protein. This represents a lower affinity binding site compared to that found for verapamil. This data can be compared to a K_D of 85 nM and a B_{max} of 120 pmol/mg protein obtained for D888 binding in carrot microsomal membranes (Graziana *et al.*, 1987) and a K_D of 1.5 nM and a B_{max} of 70 pmol/mg protein for D888 binding in skeletal muscle T-tubule membranes, (Goll *et al.*, 1984b). Both plant tissues appear to have a high density of D888 binding sites compared to skeletal muscle, but maize membranes appear to bind D888 with lower affinity compared to carrot microsome and skeletal muscle membranes. The plot possibly indicates the presence of 2 classes of low affinity binding sites for D888. If two sets of binding

sites for D888 do exist in maize coleoptile membranes, this is in contrast to the single class of D888 binding sites found in carrot microsome membranes and skeletal muscle t-tubule membranes.

Displacement of [^3H] verapamil and [^3H] D888 by a range of unlabelled antagonists was carried out to investigate whether separate binding sites for the different calcium channel antagonists exist in plant membranes. Only D600 and verapamil could significantly displace [^3H] verapamil over the concentration range tested. This suggests that D600 may be binding to the same or very similar sites as verapamil. D888 was less effective in displacing the [^3H] verapamil. This was surprising as D888 is structurally closely related to verapamil i.e. another phenylalkylamine, and had been expected to bind to the same sites as verapamil. Nifedipine, which belongs to a different structural group than verapamil i.e. the dihydropyridines, also had very little displacement effect. This suggested that nifedipine was binding to a site distinct from that binding verapamil. Displacement of [^3H] D888 by unlabelled D888, D600 and verapamil was extremely poor, despite all these drugs belonging to the same structural family. Only at the highest concentration tested was significant displacement of [^3H] D888 observed and this was lower than that seen for the displacement of [^3H] verapamil by the same concentration of unlabelled antagonist. On the whole, these results support the idea

that there are distinct binding sites for dihydropyridines and phenylalkylamine derivatives in plant membranes. It is possible that the different binding sites may represent different calcium channels which have differing sensitivities to the various antagonists. Alternatively, the separate binding sites for the different calcium channel antagonists may exist within a single channel. The latter situation has been found in animal tissues such as skeletal muscle. Both direct labelling and photoaffinity labelling experiments with a variety of channel antagonists have shown that there are distinct drug binding domains and that these binding sites are located on the α_1 subunit of the purified calcium channel (Ferry & Glossmann, 1982a; Glossmann *et al.*, 1984; Galizzi *et al.*, 1984b; Goll *et al.*, 1984a,b; Ferry *et al.*, 1984a,b; Schmid *et al.*, 1986a,b; Striessnig *et al.*, 1986a, 1987; Vaghy *et al.*, 1987).

The [^3H] verapamil binding data found for the different tissue types examined in this research is summarised in Table 3.1. together with [^3H] verapamil binding data obtained by other groups in both animal and plant tissue for comparison. The figures indicate that dissociation constants and maximum number of binding sites vary between different plant tissues and between plant and animal tissues. This suggests that the number of calcium channels in different tissues varies and indicates antagonist specificity according to tissue

type. However, the dissociation constants for plant and animal tissues presented here are all in the nanomolar range, which suggests that there is some degree of similarity in drug sensitivity between plant and animal calcium channel proteins. Animal tissues appear to have higher affinity binding sites than those found for plant tissue, possibly because more rapid responses are required in animal tissues like skeletal and cardiac muscle, than in plant tissues. On the other hand plant tissues appear to have higher densities of binding sites compared to animal tissue. For example, more channels appear to be found in maize root membranes compared to other plant tissues and to skeletal muscle membranes (Galizzi *et al.*, 1984). The apparently high density of calcium channels seen in maize roots rather than maize coleoptiles, may be found because the root is the major site of calcium uptake in the plant.

From the results of [^3H] verapamil binding to membrane fractions prepared by phase partitioning, more channels would appear to be found in the plasma membrane compared to organelle membranes. This suggests that there is subcellular localisation of verapamil binding calcium channels on plasma membrane in plants. This parallels work by Andrejauskas *et al.* (1985), which also demonstrated [^3H] verapamil binding sites to be primarily found in the plasma membrane with some sites in the ER and possibly the tonoplast. The results also indicate that binding of [^3H] verapamil by plasma membrane varies

according to tissue type. Plasma membrane from coleoptiles plus leaf rolls bound the most verapamil and plasma membrane from roots bound the least. This contradicts data presented in section 3.8.1., where roots were shown to have more verapamil binding sites than coleoptiles including leaf rolls. However in those experiments verapamil binding to total membrane was examined, whereas the above comparisons reflect verapamil binding to plasma membrane only.

When interpreting the data for [^3H] verapamil binding to the plasma membrane enriched phase and the residual membrane phase, it must be taken into consideration that the purity of the plasma membrane in the upper phase was not checked by the use of membrane marker assays and contamination with other membranes may have occurred. However contamination of the plasma membrane enriched phase is generally considered to be low (Larsson, 1983). It is also recognised that expressing the results on a mg protein basis may not give a useful estimate of the distribution of verapamil binding, as calcium channel density per unit surface area of membrane is probably the more appropriate parameter. However, expressing the results on a unit area basis would be difficult as there are no means of calculating this. To make a better comparison of the localisation of calcium channels according to membrane type, pure membrane preparations would have to be obtained and [^3H] verapamil binding examined for each membrane type.

CHAPTER 4.

THE PARTIAL PURIFICATION OF A VERAPAMIL BINDING PROTEIN FROM MAIZE COLEOPTILES.

4.1. Introduction.

The next aim of the project was to attempt to purify the verapamil binding protein from maize coleoptile membranes. This tissue was chosen as it had been found to be the richest source of high affinity verapamil binding sites and hence presumably, calcium channels. The Scatchard plot of [^3H] verapamil binding in maize coleoptile membranes indicated that there was a single class of verapamil binding sites, which suggested that there may be only one verapamil binding protein.

The voltage-dependent calcium channel has been purified from skeletal muscle using the specific binding of various calcium antagonist dihydropyridines to follow the course of purification. Two main methods have been used (Glossmann & Ferry, 1985). In the first method [^3H] nitrendipine was mixed with T-tubule membranes to form a [^3H] nitrendipine receptor complex. This complex was then solubilised with digitonin and purified 330 fold by a combination of lectin affinity chromatography, ion exchange chromatography and sedimentation through sucrose gradients (Curtis & Catterall, 1984). In the second method, the dihydropyridine receptor was solubilised in CHAPS and then separated using anion exchange, lectin affinity and gel filtration chromatography, to give an 80

fold purification of the dihydropyridine receptor. [³H] (+)-PN 200-110 was used to detect the purified receptor by direct binding experiments after each purification step (Borsotto *et al.*, 1985). The second method has one major advantage over the first in that it does not rely on the formation and maintenance of a antagonist-receptor complex and therefore avoids potential problems related to the dissociation of the antagonist-receptor complex during purification. Using these methods, the structure of the skeletal muscle antagonist receptor was determined and it was shown to be a functional calcium channel (Curtis & Catterall, 1984,1985,1986; Borsotto *et al.*, 1985; Flockerzi *et al.*, 1986a,b; Striessnig *et al.*, 1986b). The channel consists of 4/5 subunits, of which the α_1 subunit is the main functional subunit and contains the drug receptor site (Takahashi *et al.*, 1987). Thus by purification of the antagonist receptor the whole channel was purified. Working on this basis it was hoped to purify the verapamil binding protein and therefore the calcium channel from plant membranes. Taking a lead from published purification methods, attempts were made to solubilise the verapamil binding protein from maize coleoptile membranes and then to purify the solubilised protein using a similar combination of chromatography techniques as described for the purification of the dihydropyridine receptor from skeletal muscle. Due to the relatively low affinity of [³H] verapamil for its binding site in maize membranes, the production of a [³H]

verapamil receptor complex was not feasible. As a result, the procedure used to purify the verapamil binding protein was based largely on the second animal channel purification method, with the purified protein being detected by direct binding experiments with [^3H] verapamil after each purification step.

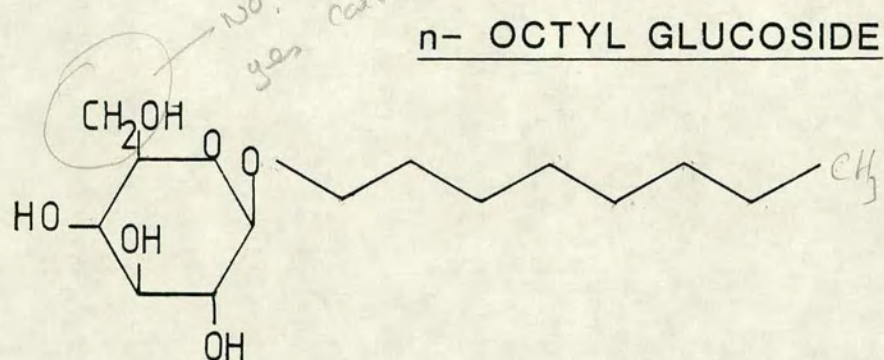
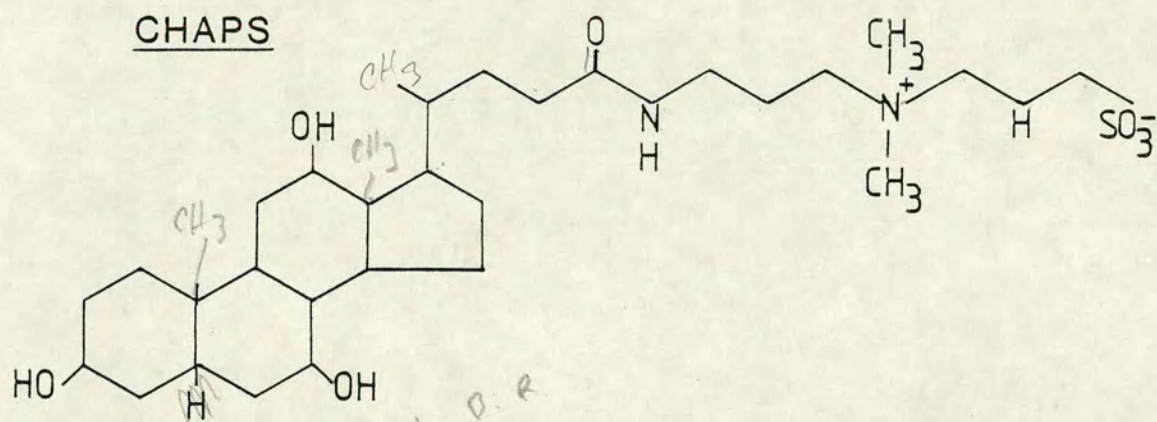
The development of the protocol adopted to solubilise and partially purify the verapamil binding protein from maize coleoptile membranes is presented in this chapter.

4.2. Solubilisation of the Membrane Bound Verapamil Binding Protein.

A pre-requisite for purifying membrane proteins by column chromatography, is that the protein in question can be solubilised in a non-denatured and relatively stable form. Experiments were therefore carried out to find the optimum conditions for solubilisation of the verapamil binding protein. The results of these investigations are presented in the following section.

4.2.1. *Optimisation of solubilisation.*

Preliminary experiments were carried out using a variety of detergents, to determine the optimum detergent type to solubilise the verapamil binding protein. These were Triton X-100, octyl-glucoside (non-ionic detergents) and CHAPS (a zwitterionic detergent). Fig.4.1. shows the molecular structures of these detergents.



TRITON X-100

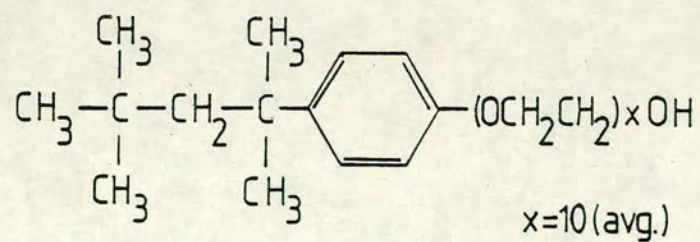


Fig.4.1. Molecular structures of the detergents CHAPS, n-octyl glucoside and Triton x-100.

The recovery of [^3H] verapamil binding activity after solubilisation, expressed as a percentage of [^3H] verapamil binding activity initially found in crude membrane fractions, is summarised in Table 4.1. Recovery of activity after solubilisation with Triton X-100 and octyl glucoside was less than 10% at all the concentrations investigated. The detergent CHAPS was more effective over the same concentration range. At 0.2% CHAPS, 16% of [^3H] verapamil binding activity was recovered. This increased to 40% when the membrane fractions were solubilised in 0.3% CHAPS. A decline in binding activity was observed after solubilisation with 0.5% and 1.0% CHAPS, with recoverys of 30% and 8% respectively.

CMC = 8 mM \Rightarrow 0.3% \downarrow 0.5 + 1% \downarrow

CHAPS was therefore selected as the detergent for further study because of its ability to solubilise the verapamil binding protein with relatively high levels of recovery of [^3H] verapamil binding activity compared to the other detergents investigated. As 0.3% CHAPS had given the best recovery of binding activity, this concentration was used initially.

The effects of 20% (w/v) sucrose or glycerol on solubilisation of the verapamil binding protein were investigated, as the addition of glycerol or sucrose to the solubilisation medium had been found to stabilise the solubilised nitrendipine receptor from rabbit skeletal muscle tubule membranes and had increased the yield of active receptor from 25% to approximately 71% (Borsotto

<u>Detergent concentration.</u>	<u>Recovery of [³H] verapamil binding activity. (% of [³H] verapamil binding in crude membrane).</u>
<u>Triton x-100</u>	
0.2 %	1.5
0.5 %	3.0
1.0 %	5.0
<u>Octyl glucoside</u>	
0.2 %	8.0
0.5 %	-
1.0 %	-
<u>CHAPS</u>	
0.2 %	16.0
0.3 %	40.0
0.5 %	30.0
1.0 %	8.0

Table 4.1. Solubilisation of [³H] verapamil binding activity using Triton x-100, octyl glucoside, Deca-10 and CHAPS.

Crude maize coleoptile membrane fractions were solubilised in a buffer containing one of the above detergents at varying concentrations. After incubation for 60 min at 4°C, the mixtures were centrifuged. The resulting supernatants were assayed for [³H] verapamil binding using the standard filter assay.

et al., 1984). The results shown in Table 4.2. indicate that the recovery of [^3H] verapamil binding activity was increased by the inclusion of 20% glycerol or 20% sucrose in the solubilisation buffer. As 20% sucrose gave the highest recovery of [^3H] verapamil binding activity, this was added routinely to the solubilisation buffer in all further experiments.

In the presence of 20% sucrose, the optimum concentration of CHAPS required to solubilise the verapamil binding protein was found to have increased. The graph shown in Fig 4.2. indicates that 0.5% CHAPS in solubilisation buffer gave the best recovery of verapamil binding activity. This equates with a protein:detergent ratio of 1:14 under the conditions used.

In all subsequent solubilisation experiments, 0.5% CHAPS and 20% sucrose in 50 mM Tris, 50 mM Mops, pH7.5 at a 1:14 protein:detergent ratio was used to solubilise the verapamil binding protein. Using these conditions the recovery of verapamil binding activity after solubilisation was between 40-60%. The average purification of the verapamil binding protein achieved after solubilisation of the crude membrane preparation was 2.6 ± 0.53 (n=9) fold. Crude membrane preparations were always solubilised immediately, as recovery of binding activity after solubilisation of a previously frozen crude membrane preparation was only about 50% of that recovered after solubilisation of a fresh preparation. It should also be noted that when

<u>Solubilisation conditions.</u>	<u>Recovery of [³H] verapamil binding activity. (% of [³H] verapamil binding to crude membrane).</u>
<u>0.3% CHAPS in 50 mM Tris 50 mM Mops pH 7.5.</u>	8
<u>0.3% CHAPS in 50 mM Tris 50 mM Mops 20% Glycerol pH 7.5.</u>	36
<u>0.3% CHAPS in 50 mM Tris 50 mM Mops 20 % Sucrose pH 7.5.</u>	41

Table 4.2. The effect of the inclusion of glycerol or sucrose on CHAPS solubilisation of verapamil binding activity.

Crude maize coleoptile membrane fractions were solubilised in buffer containing 0.3% (5 mM) CHAPS \pm 20% sucrose or glycerol. After incubation for 60 min at 4⁰C the mixtures were centrifuged. The supernatants were assayed for [³H] verapamil binding using the standard filter assay.

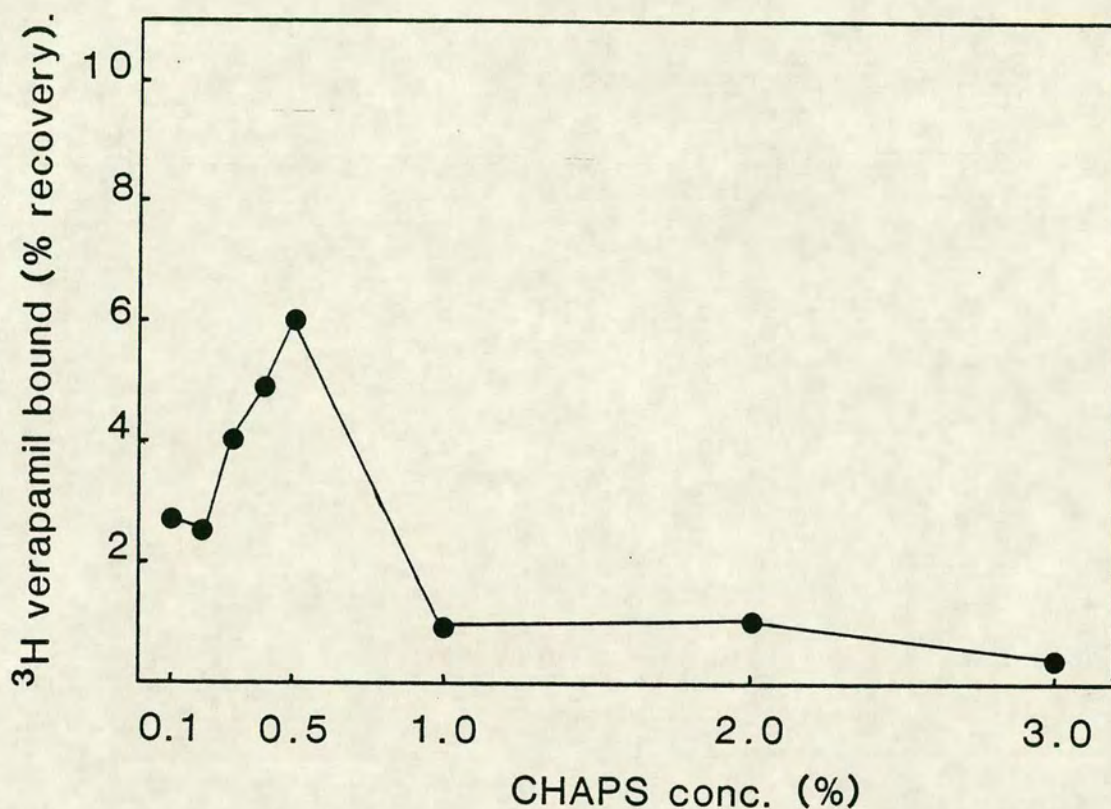


Fig.4.2. Determination of the optimum concentration required for CHAPS solubilisation of [^3H] verapamil binding activity.

Crude maize coleoptile membrane fractions were solubilised in buffer containing a range of CHAPS concentrations, plus 20% sucrose. After the standard solubilisation procedure, the supernatant and the residual pellet were assayed for [^3H] verapamil binding using the standard filter assay.

solubilised membrane fractions were frozen in liquid nitrogen and stored at -70°C , recovery of binding was also reduced by about 50%. For this reason solubilised membrane fractions were kept at 4°C and used within several hours, over which time the loss of binding activity was negligible.

It was also observed that there was a high proportion (68% on average), of verapamil binding recovered in the insoluble protein fraction i.e. the pellet, at every concentration of CHAPS investigated. This activity may represent binding of [^3H] verapamil to CHAPS insoluble binding sites or the entrapment of [^3H] verapamil in vesicles during centrifugation. At high concentrations of CHAPS (1.0%, 2.0%, 3.0%) the percentage recovery of binding in the solubilised fraction decreases but remains high in the insoluble fraction. It may be possible that at these high concentrations of CHAPS the verapamil binding sites are denatured, so that even if they are still present in the supernatant they cannot bind verapamil. At the lower concentrations of CHAPS (0.1%-0.3%), there may not be enough CHAPS present to solubilise as many binding sites as 0.4%-0.5% CHAPS, thus more verapamil binding activity is seen in the pellet and less in the solubilised fraction.

4.2.2. [^3H] verapamil binding in CHAPS solubilised membrane fractions.

Using the standard [^3H] verapamil binding assay, the binding characteristics of the labelled antagonist were examined in CHAPS solubilised membrane fractions.

Fig.4.3. shows a typical displacement curve for [^3H] verapamil binding in CHAPS solubilised membrane fractions. The graph is similar to that obtained for [^3H] verapamil binding in crude membrane preparations. Increasing concentrations of unlabelled verapamil are able to displace more labelled verapamil. At 100 μM unlabelled verapamil, [^3H] verapamil is displaced by approximately 93%. The IC_{50} is about 700 nM and can be compared to the IC_{50} of 100 nM found in crude membrane preparations.

Using this data, a Scatchard plot of [^3H] verapamil binding in solubilised membrane fractions was constructed (Fig 4.4.). In the CHAPS solubilised preparation the K_D was 158 nM and the B_{max} was 78 pmol/mg protein. These values can be compared to those obtained for [^3H] verapamil binding to crude membrane preparations, where the K_D was 72 nM and the B_{max} was 135 pmol/mg protein. In both cases the Scatchard plots were linear which suggests the presence of a single class of non-interacting binding sites. From the similarities in binding curves and Scatchard plots, it is concluded that the solubilised verapamil binding protein has similar properties to those

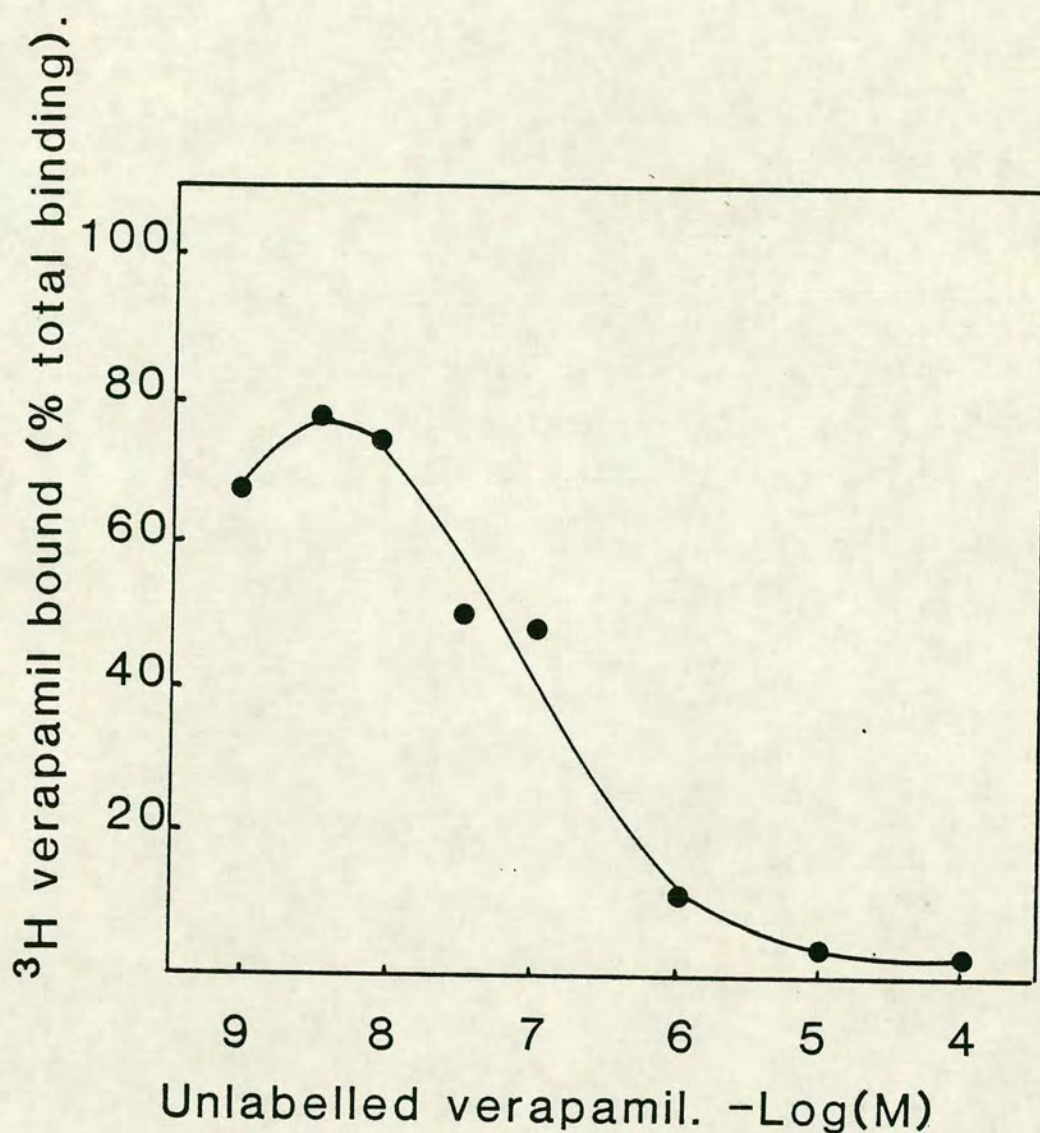


Fig.4.3. Displacement of [³H] verapamil by unlabelled verapamil in CHAPS solubilised maize coleoptile membrane fractions.

CHAPS solubilised membrane fractions (0.5 - 1.0 mg/500 μ l assay) were incubated with [³H] verapamil to give a final concentration of 2.24 nM. Unlabelled verapamil was added at the concentrations indicated. The amount of bound [³H] verapamil was determined using the standard filter assay. Total [³H] verapamil binding was 120 c.p.m.

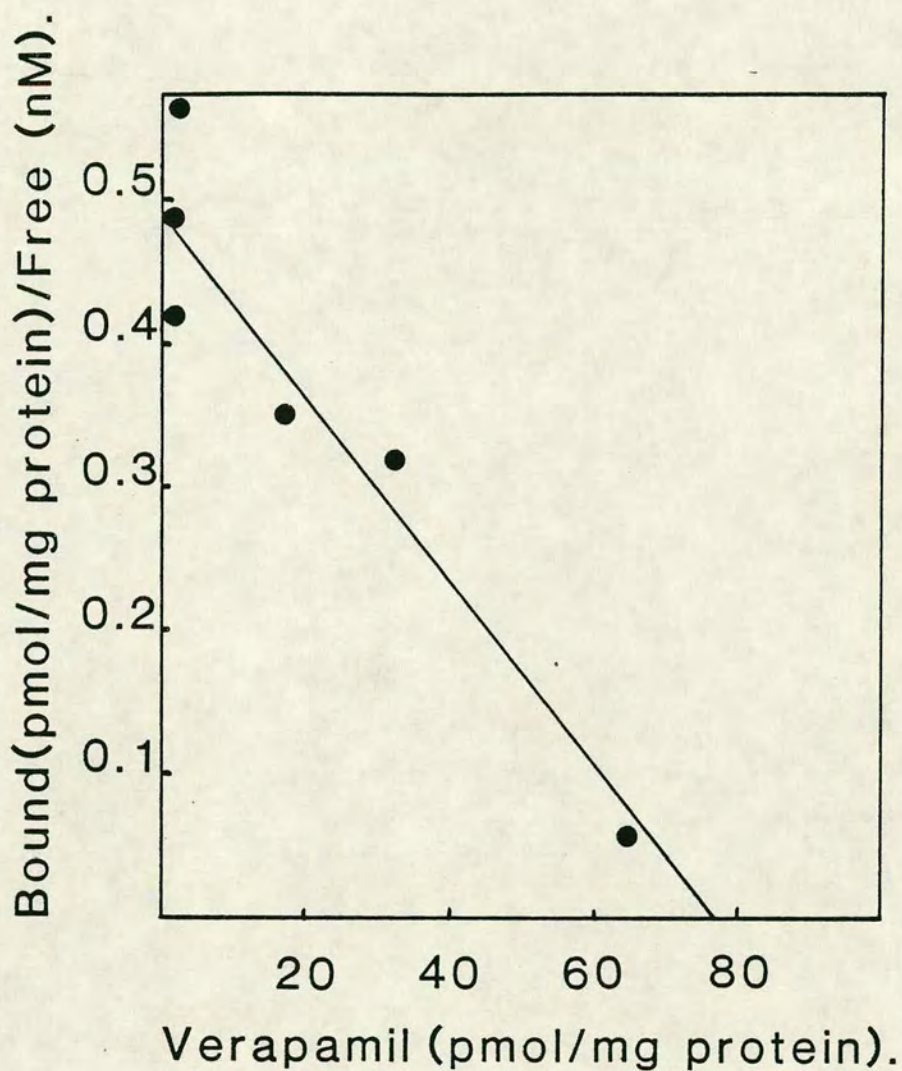


Fig.4.4. Scatchard plot of [³H] verapamil binding to CHAPS solubilised maize coleoptile membrane fractions.

Data from Fig.4.2. was transformed to give a Scatchard plot. Least squares analysis was used to plot the line of best fit. The apparent K_D was 158 nM and the B_{max} was 78 pmol/mg protein.

of the membrane bound protein. However, it should be noted that [^3H] verapamil binding to the solubilised protein is of slightly lower affinity compared to the membrane bound protein. A loss in binding capacity may be due to an adverse effect on the verapamil binding protein caused by the solubilisation procedure. This in turn may effect the subsequent purification steps.

4.3. DEAE Ion Exchange Column Chromatography..

Following solubilisation of the verapamil binding protein from its membrane bound form, further purification of the verapamil binding protein was attempted using anion exchange column chromatography. DEAE anion exchange was selected as the first purification step for several reasons. 1. the solubilised sample volume was very large (approximately 20-30 ml), which prevented the easy use of gel filtration chromatography as a first step. 2. DEAE anion exchange provides an opportunity to remove large groups of negatively charged proteins and lipids, thereby removing a large component part that would bind verapamil in a non-specific manner. DEAE ion exchange was also chosen as the first step in the purification of the dihydropyridine receptor from rabbit skeletal muscle (Borsotto *et al.*, 1984).

4.3.1. Development of the elution protocol.

The optimum conditions required for the elution of the verapamil binding protein were investigated in a number of pilot experiments using linear and step gradients of varying ionic strength.

Fig.4.5. shows the elution profile of the DEAE column utilising a 0-0.5 M NaCl linear gradient. The graph indicates that a small amount of verapamil binding activity, with fairly low specific activity, was eluted from the column during loading of the sample and during the subsequent wash step. The major part of verapamil binding activity was eluted from the column over the first part of the gradient, upto approximately 0.2 M NaCl. Recovery of binding activity decreased as the gradient concentration increases, apart from a small rise in activity towards the end of the gradient (0.5 M NaCl). The major peak of verapamil binding activity was eluted with the major protein peak.

In subsequent experiments the DEAE column was eluted stepwise with 0.1 M and 0.2 M NaCl. Fig.4.6. shows a typical elution profile using this protocol. The graph indicates that most of the verapamil binding activity is eluted from the column with 0.1 M NaCl and this binding activity has a relatively high specific binding activity. Approximately 16% of the binding activity initially loaded onto the column was recovered with this step, compared to only 4% with 0.2 M NaCl. Total recovery of

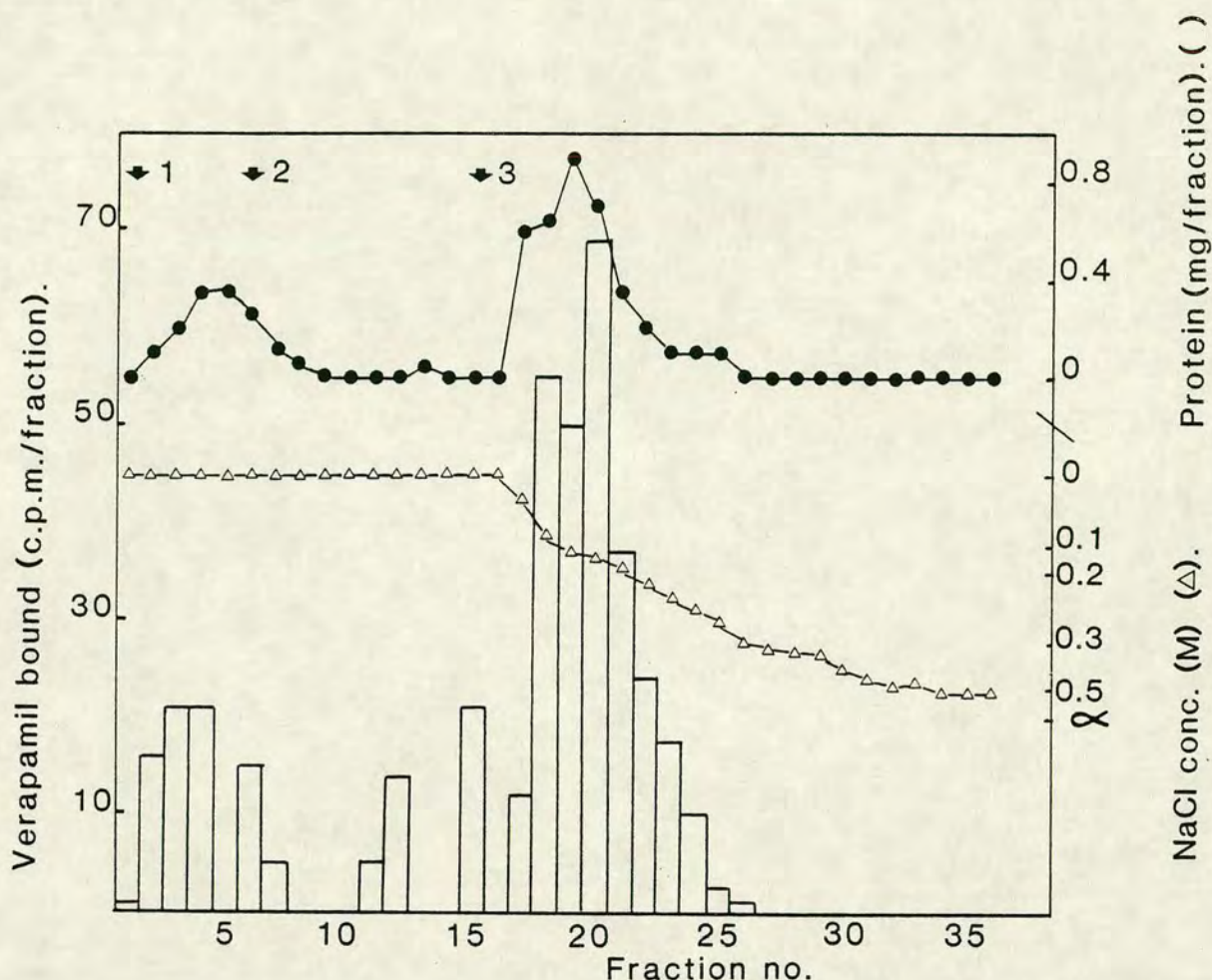


Fig.4.5. DEAE column elution profile using a 0 - 0.5 M NaCl linear gradient.

Approx. 10 ml of solubilised membrane fraction was loaded onto a pre-equilibrated 4 x 1 cm DEAE column (1). The column was washed with 10 ml of column buffer (2). The verapamil binding activity was then eluted from the column using a linear gradient of 0 - 0.5 M NaCl in column buffer (3). Fractions of 2 ml were collected and assayed for [^3H] verapamil binding. In a separate experiment, the NaCl gradient was determined by taking a conductivity reading for each fraction and for fractions containing a known concentration of NaCl.

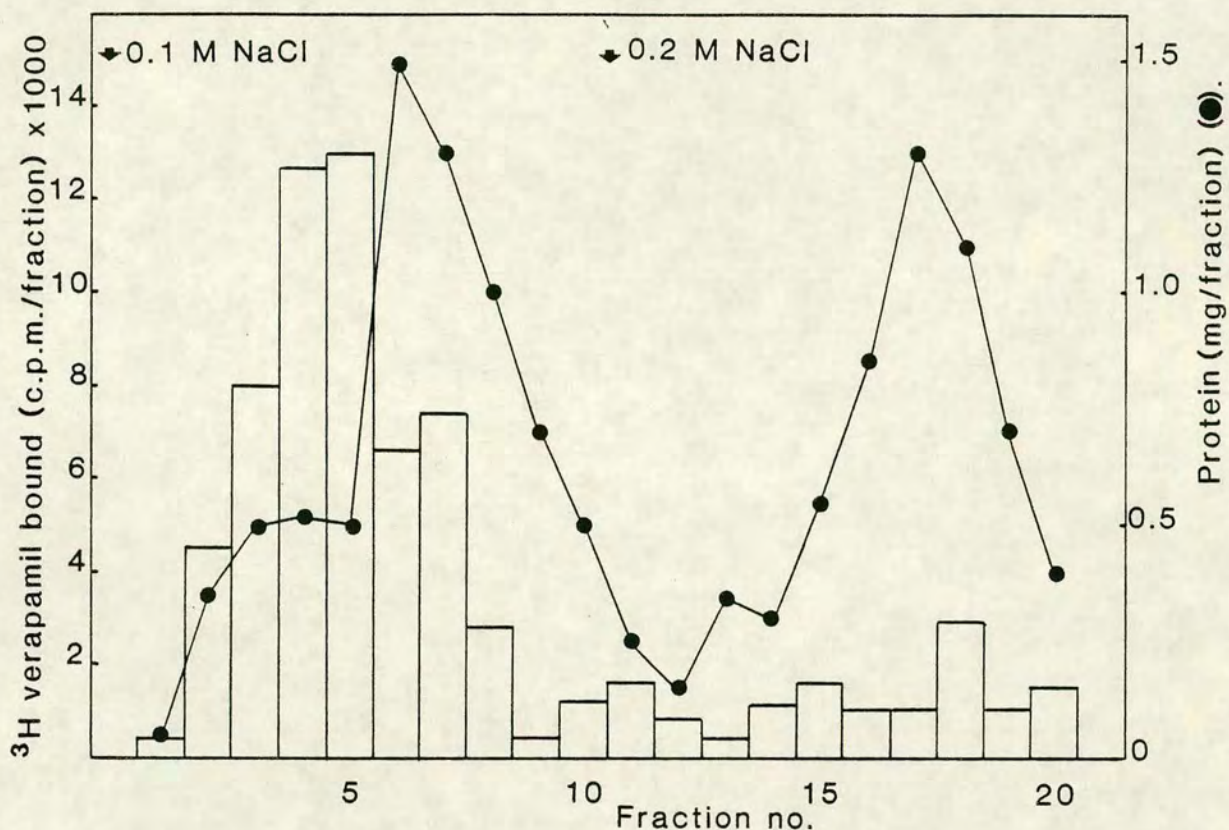


Fig.4.6. DEAE column elution profile using a 0.1 M and 0.2 M NaCl step gradient.

Approx. 10 ml of solubilised membrane fraction was loaded onto a pre-equilibrated 4 x 1 cm DEAE column which was then washed with 10 ml of column buffer. The verapamil binding activity was eluted from the column using 0.1 M and 0.2 M NaCl steps, as indicated on the graph. Fractions of 1 ml were collected and assayed for [³H] verapamil binding.

binding activity was 20% (before correction), indicating a loss in binding activity of upto 80%. On average the loss in binding activity incurred over the duration of the DEAE column run was $42.5 \pm 15\%$ ($n=7$). This accounts for a large proportion of the loss in activity observed here. In further experiments in which the loss of activity was measured, the figures for the overall recovery of binding activity have been corrected to take this loss into account.

After elution from the DEAE column with 0.1 M NaCl, the verapamil binding protein was purified from the solubilised membrane preparation by just over 1 fold. (3 fold purification from crude membrane fractions). In similar experiments carried out using this protocol, it was often observed that recovery of verapamil binding activity, with a relatively high specific activity, was not always eluted with 0.1 M NaCl step. Bearing this in mind, it was decided to elute the verapamil binding protein from the DEAE column with 0.15 M NaCl. This was in order to elute as much verapamil binding activity as possible, while at the same time avoiding the dilution of the verapamil binding activity with proteins with no or very little binding activity.

4.3.2. The final elution protocol.

As a result of the above experiments, the solubilised verapamil binding protein was applied to the DEAE column and eluted with 0.15 M NaCl. This was carried

out routinely as the first chromatographic step in the purification of the verapamil binding protein.

When the dihydropyridine receptor was purified from rabbit skeletal muscle it was found that the inclusion of a low percentage of detergent was necessary to obtain a good percentage recovery of binding activity. For this reason 0.1% CHAPS was included in the 0.15 M NaCl elution buffer used for the DEAE column (and in all column buffers used in the final purification protocol). 20% sucrose was also present in the elution buffer to maintain the stability of the protein during purification.

Using this method, the total fold purification of the verapamil binding protein after solubilisation of the crude membrane fraction and subsequent fractionation on a DEAE column was on average 3.37 ± 1.41 ($n=9$) fold. Fractionation of the solubilised membrane preparation on a DEAE column gave an average fold purification of 2.25 ± 1.28 ($n=9$). Recovery of binding activity after DEAE column chromatography of the solubilised fraction was $69 \pm 42\%$ ($n=9$) of that initially loaded. Recovery of protein was 14.5 ± 2.3 mg or $31 \pm 5.6\%$ ($n=9$).

Although the purification factor achieved with the DEAE column was fairly low (2.25 ± 1.28 $n=9$), the DEAE step was retained in the purification procedure in order to remove components of the solubilised sample (lipid, protein, pigment complexes), which would otherwise prevent the success of subsequent purification steps.

4.4. Gel Filtration Column Chromatography.

Gel filtration column chromatography was employed for two reasons. 1. To purify further the verapamil binding protein found in the peak binding fractions eluted from the DEAE column. 2. To obtain an estimation of the molecular weight of the verapamil binding protein/complex.

In preliminary purification investigations, it had been noticed that decay of binding activity occurred over time. Therefore attempts were made to minimise denaturation of the verapamil binding protein by using a high flow rate gel filtration matrix. Sephacryl S300 and S400 were both examined chosen as Sephacryl gives higher flow rates than most other gel filtration matrices. Sephacryl S300 was used in initial experiments, but discarded in favour of S400 in later (and in the final purification procedure), in an attempt to obtain a more accurate estimation of the molecular weight of the verapamil binding protein.

4.4.1. *Sephacryl S300 experiments.*

DEAE fractions containing the highest verapamil binding activity were fractionated on a Sephacryl S300 column.

Fig.4.7.a. shows a typical S300 column elution profile. The graph indicates that the verapamil binding activity was eluted from the column as one main peak. The

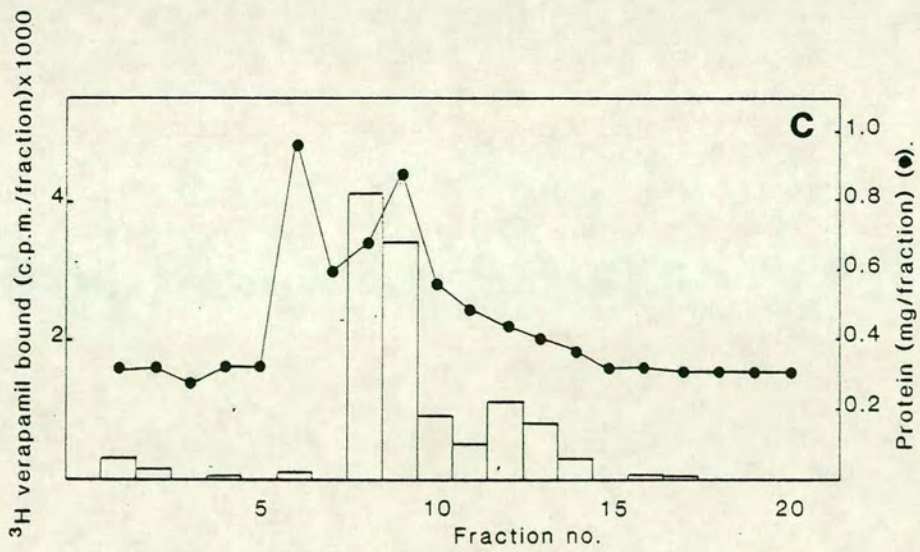
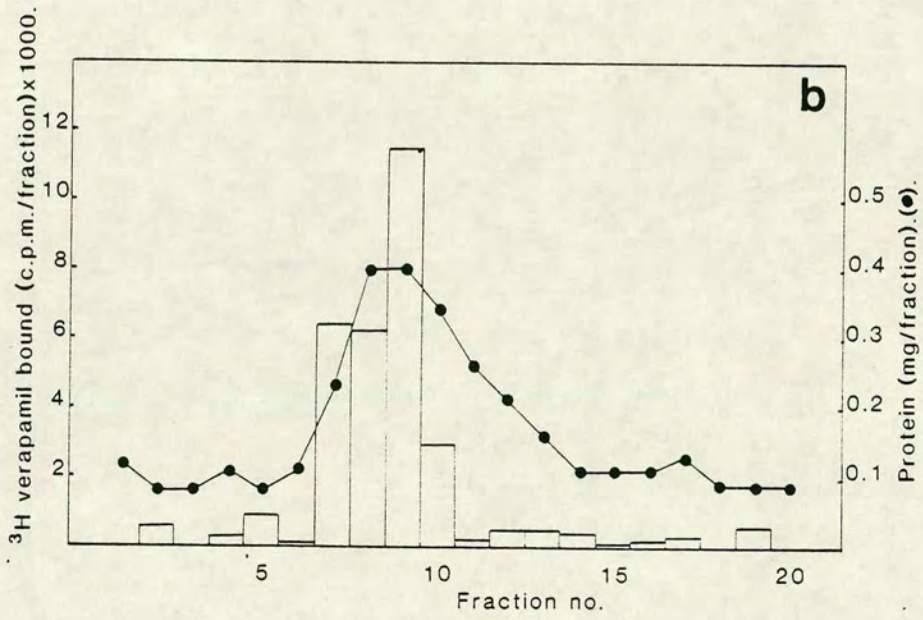
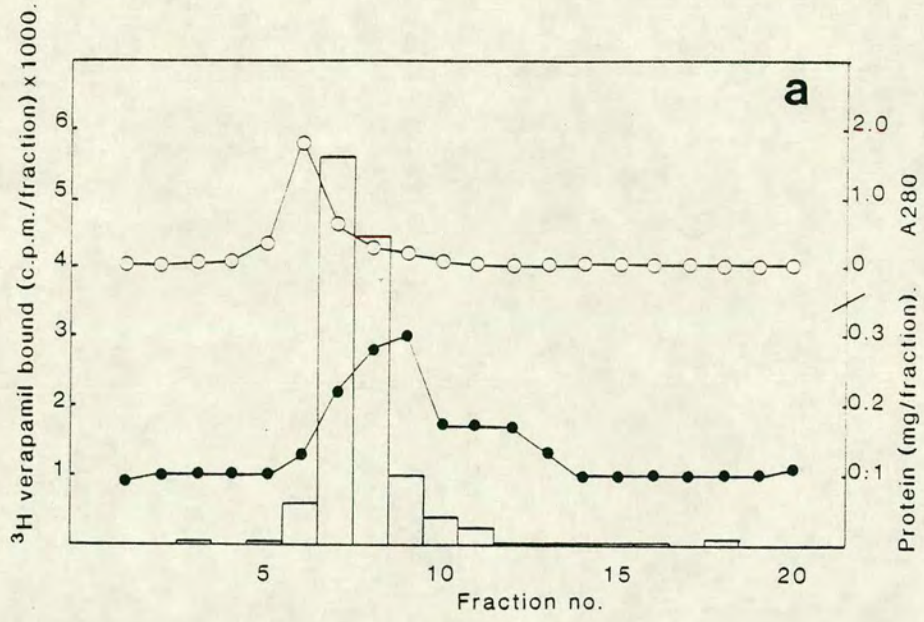
Fig.4.7.a.b.c. Sephacryl S300 column elution profiles.

Approx. 0.5 ml of pooled peak verapamil binding fractions from the DEAE column were loaded onto a pre-equilibrated Sephacryl S300 column.

The column was eluted with :-

- a. column buffer.
- b. column buffer plus 1 M NaCl.
- c. column buffer plus 1 mM DTT.

Fractions of 1 ml were collected and assayed for [^3H] verapamil binding. The void volume of the column was determined separately using Blue Dextran 2000 monitored at A_{280} .



elution position corresponds with the elution of the Blue Dextran 2000 from the column, indicating that the verapamil binding protein is eluted in the void volume of the column. This suggests that the verapamil binding protein or verapamil binding protein complex has a molecular weight of greater than 1.5×10^6 Daltons.

To investigate whether the verapamil binding protein was being eluted from the column as a large aggregate, DEAE fractions were separated on S300 columns in the presence of 0.2 M, 1.0 M NaCl or 1 mM DTT in order to break up the potential aggregate. High salt was added to reduce protein/protein ionic interactions and DTT to break disulphide linkages between proteins. However, the inclusion of these substances in the elution buffer did not significantly alter the elution behaviour of the verapamil binding activity. In each case the verapamil binding activity was eluted in the void volume of the column. Figs. 4.7. b. & c. illustrate Sephacryl S300 column elution profiles in the presence of 1 M NaCl and 1 mM DTT.

The average purification obtained after S300 fractionation of peak DEAE fractions was 13 ± 7 fold ($n=4$). The variation in purification may be attributed to the extent of purification achieved in earlier steps and may possibly depend on the presence of NaCl and DTT, but this was not investigated further. The recovery of binding activity from the column ranged from 29% to over 100% of that originally applied to the column.

4.4.2. Sephacryl S400 experiments.

The results obtained from the Sephacryl S300 experiments suggested that the verapamil binding protein may be a large complex with a molecular weight of over 1.5×10^6 Daltons. To investigate this further a Sephacryl S400 column, with a fractionation range of $2 \times 10^4 - 8 \times 10^6$ Daltons, was employed.

A typical S400 column elution profile is shown in Fig.4.8. The graph shows that the verapamil binding activity is eluted from the column in the void volume, as indicated by the Blue Dextran 2000. This suggests that the verapamil binding protein is larger than first thought and chromatographs as a large aggregate with a molecular weight of over 8 million daltons.

Phenyl Sepharose separation of the DEAE fraction prior to gel filtration fractionation, also had no effect on the elution behaviour of the verapamil binding protein, which still eluted in the void volume.

Purification of the verapamil binding protein, after application of DEAE fractions containing peak verapamil binding activity to a Sephacryl S400 column, was on average 13.5 ± 8.17 fold ($n=9$). The average total purification i.e. the purification obtained from crude membrane, was 22.5 ± 9.59 fold ($n=9$). Data from a number of experiments indicated that the recovery of binding activity in the pooled peak fraction was over 100% of that originally loaded onto the column. This increase in

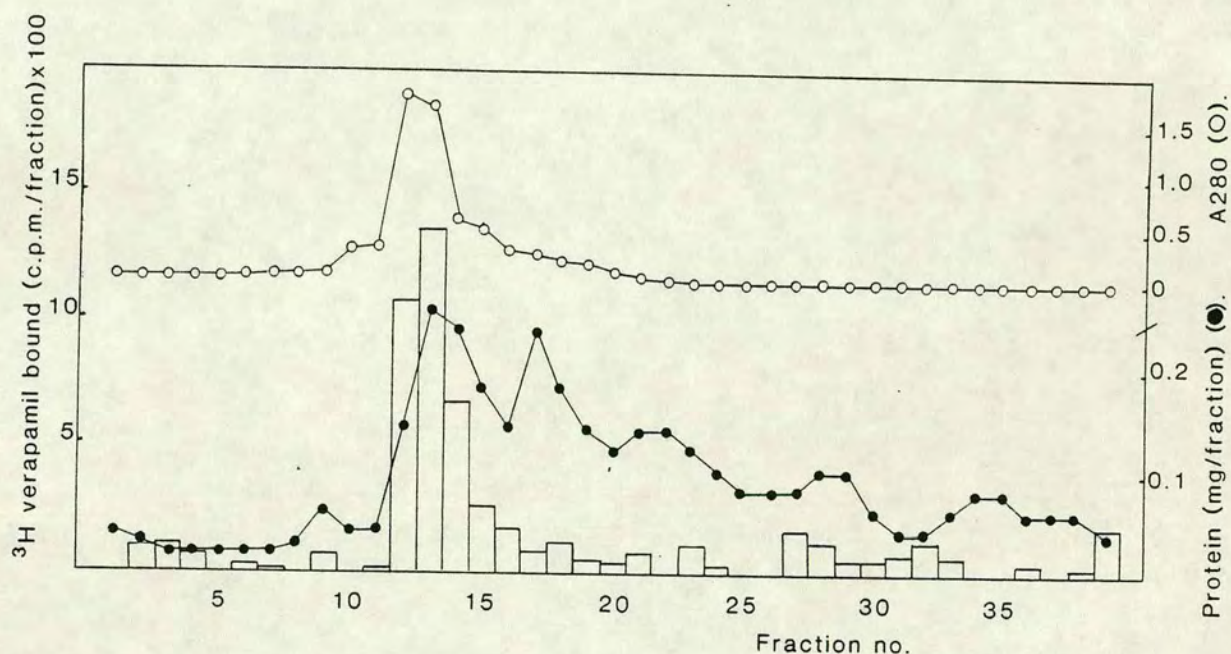


Fig.4.8. Sephacryl S400 column elution profile.

Approx. 2 ml of pooled peak verapamil binding fractions from the DEAE column were loaded onto a pre-equilibrated Sephacryl S400 column. The column was then eluted with column buffer. Fractions of 1 ml were collected and assayed for [^3H] verapamil binding. The void volume of the column was determined separately using Blue Dextran 2000 monitored at A_{280} .

binding activity, also seen in the S300 experiments, may be due to the removal of inhibitory binding proteins during purification and/or by the purification of other proteins which may promote verapamil binding. Recovery of protein was 2.5 ± 1.0 mg or $16.1 \pm 4.7\%$ ($n=9$).

The figures quoted here have been adjusted for losses in activity which occurred due to freezing of the DEAE fraction prior to application to the S400 column and those losses incurred over the duration of the DEAE column run. On average the loss in activity due to freezing was $51 \pm 10.8\%$ ($n=5$). In some instances no activity appeared to be lost due to freezing. The average loss in activity over the duration of the DEAE column run was $42.5 \pm 15\%$ ($n=7$), (see section 4.3.2.). (Adjustments were made for each individual experiment before the results were averaged). Losses in binding activity during the S400 column run were negligible.

4.5. Hydrophobic Interaction Column Chromatography.

Following partial purification of the verapamil binding protein using ion exchange and gel filtration column chromatography, further purification was attempted using hydrophobic interaction chromatography (HIC). This type of chromatography was chosen because it is known that calcium channels are hydrophobic in nature. Phenyl Sepharose CL-4B was chosen as the column matrix in preference to octyl Sepharose, as strong and weak

hydrophobic proteins can be eluted fairly easily from this matrix.

In initial experiments phenyl Sepharose was used to separate fractions containing peak verapamil binding activity eluted from the DEAE column. Once suitable conditions for obtaining peak verapamil binding activity from the phenyl Sepharose column had been determined, fractions from the Sephacryl S400 column were separated on phenyl Sepharose columns using similar conditions.

The following section presents the development of the phenyl Sepharose column elution protocol and the purification of the verapamil binding protein from S400 fractions achieved with the final optimum elution protocol.

4.5.1. Development of the elution protocol.

A number of elution protocols were utilised to optimise the conditions required for 1. Loading of the sample onto the column. 2. Washing of the column to remove non-specific binding proteins. 3. Elution of the verapamil binding protein with a high specific activity. Optimum conditions were investigated by varying the salt, calcium, detergent/sucrose concentration and buffer components of the load, wash and elution buffers. These changes alter the hydrophobicity of the aqueous environment of the column matrix and consequently affect the behaviour of the verapamil binding protein and other proteins at the load, wash and elution steps. A balance

between the recovery of total binding activity and an increase in specific activity was looked for with each elution protocol, the aim being to obtain the verapamil binding protein in a fraction with high specific activity and most of the binding activity. (See Table 4.3.).

The elution profiles for the different protocols investigated are shown in Fig.4.9. - Fig.4.13. Essentially three main buffers were used in the elution protocols. These were either 1. High salt, high Ca^{2+} , high detergent & sucrose, Tris & Mops, referred to as high salt buffer. 2. Low salt, high Ca^{2+} , detergent & sucrose, Tris & Mops, referred to as low salt buffer. 3. Low detergent & sucrose, high salt, high Ca^{2+} , Tris & Mops, referred to as low detergent/sucrose buffer. The sample was loaded onto the column and washed in one of the above buffers. The column was then eluted by the removal of salt, Ca^{2+} or Tris & Mops from the basic buffer, as indicated in the figures. These buffers are referred to as low salt elution buffer, low calcium elution buffer, minus Tris/Mops elution buffer. Minus Ca^{2+} elution buffer refers to an elution step of unbuffered EGTA only.

Fig.4.9. illustrates the elution profile for the phenyl Sepharose column using protocol 1. Essentially this protocol involved loading and washing of the column in low salt buffer, followed by sequential elution with low calcium and minus Tris/Mops elution buffer. Total recovery of binding activity using this protocol was 90%

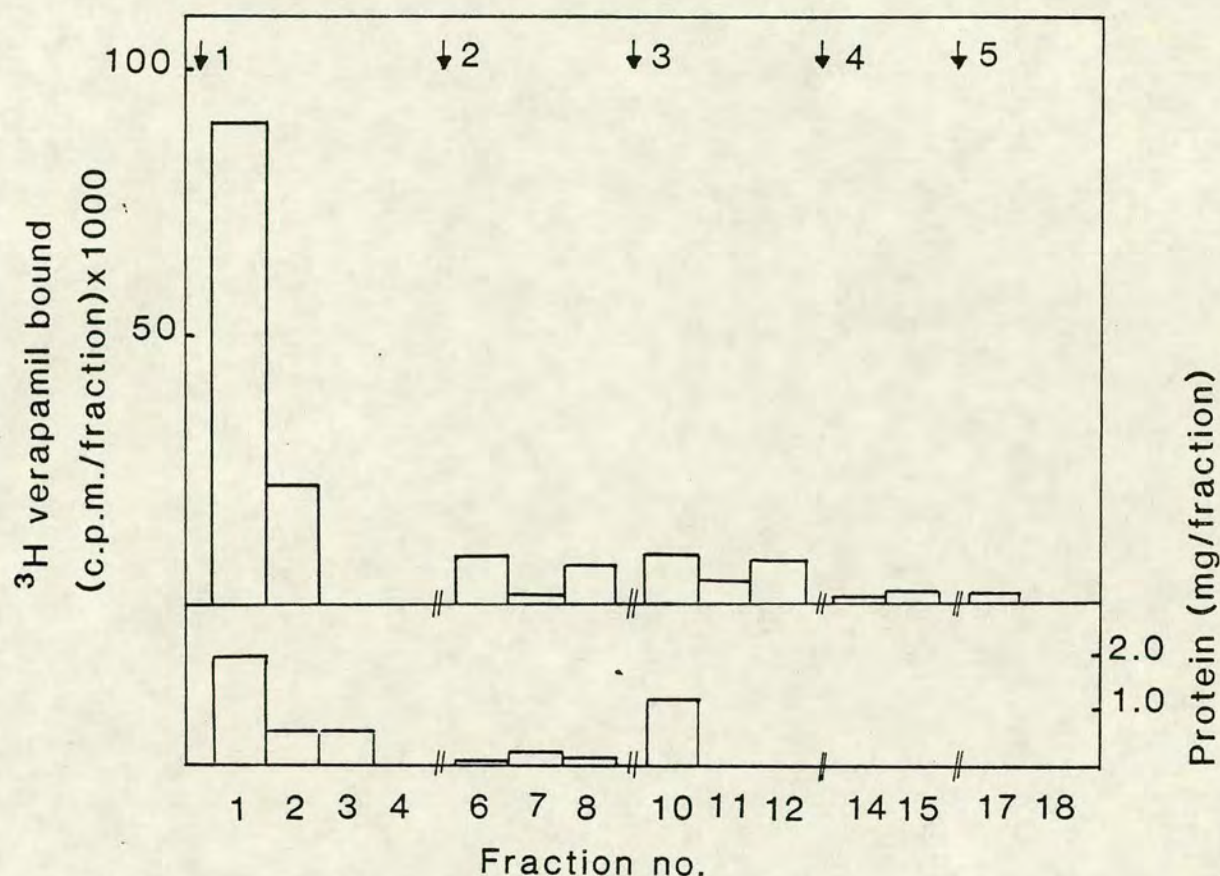


Fig.4.9. Phenyl Sepharose column elution profile using protocol 1.

Approx. 4 ml of pooled peak verapamil binding fractions from the DEAE column were adjusted to 1 mM CaCl_2 and loaded onto a 4 x 1 cm phenyl Sepharose column which had been pre-equilibrated with low salt buffer. The column was then washed with low salt buffer (1), followed by low Ca^{2+} buffer (2), minus Tris/Mops buffer (3), minus Ca^{2+} buffer (4) and d. H_2O (5). Fractions of 4 ml were collected and assayed for [^3H] verapamil binding.

An A_{280} trace was used to indicate protein elution from the column. Fractions were collected when the trace deviated from a basal level. Fractions containing no protein were not collected and are indicated by // on the graph.

Table 4.3. Recovery of [^3H] verapamil binding activity and specific activity for each phenyl Sepharose elution step. Protocol 1-5.

LOAD/WASH CONDITIONS.	ELUTION CONDITIONS.							
	WASH.	LOW SALT.	LOW Ca^{2+} .	MINUS TRIS/HOPS. (low D/S).	MINUS TRIS/HOPS. (high D/S).	MINUS Ca^{2+} .	H ₂ O.	% TOTAL ACTIVITY RECOVERED.
1. LOW SALT	% REC.	71	-	12	-	14	2	90%
	S.A.	35272	-	40000	-	18567	ND	
2. HIGH SALT	% REC	2	6	19	-	72	1	32%
	S.A.	2028	1488	25500	-	12922	ND	
3. LOW D/S	% REC	50	1.3	-	32	17	-	83%
	S.A.	10594	696	-	18096	246500	-	
4. HIGH SALT	% REC	14	10	-	8	68	-	35%
	S.A.	1950	757	-	5050	105280	-	
5. HIGH SALT	% REC	13	39	10	-	34	-	46%
	S.A.	8181	35866	34500	-	7966	-	

% Total activity recovered indicates the activity recovered from the column as a percentage of that initially loaded. % Rec. indicates the recovery of [^3H] verapamil binding activity for each elution step as a percentage of the total binding activity recovered from the column. SA indicates the specific activity of each elution step. D/S denotes detergent/sucrose.

of that originally loaded onto the column. Of this activity, 71% was eluted from the column during the low salt loading and washing stage and had a relatively high specific activity. Low calcium elution buffer followed by detergent/sucrose only elution, both eluted smaller amounts of binding activity, but of fairly high specific activity. No further elution of protein or binding activity was achieved with either minus Ca^{2+} buffer or H_2O . (Table 4.3.).

Due to the high loss of binding activity under low salt loading conditions, the inclusion of high salt in the loading and washing steps was investigated in protocol 2. In summary, following the high salt load and wash, the column was eluted with low salt, low Ca^{2+} and minus Tris/Mops elution buffers (Fig.4.10.). Using this protocol 32% of the binding activity initially loaded onto the column was recovered. The graph indicates that in the presence of salt a high proportion of the verapamil binding activity remained bound to the column. Only 2% of the total binding activity recovered was washed off the column at this stage. (Table 4.3.). After low salt elution very little binding activity eluted from the column. Low Ca^{2+} eluted a small amount of activity but with high specific activity. However, the majority of the verapamil binding activity with relatively high specific activity was eluted with minus Tris/Mops elution buffer. Very little activity or protein was eluted after this by the removal of Ca^{2+} (minus Ca^{2+} buffer) or H_2O .

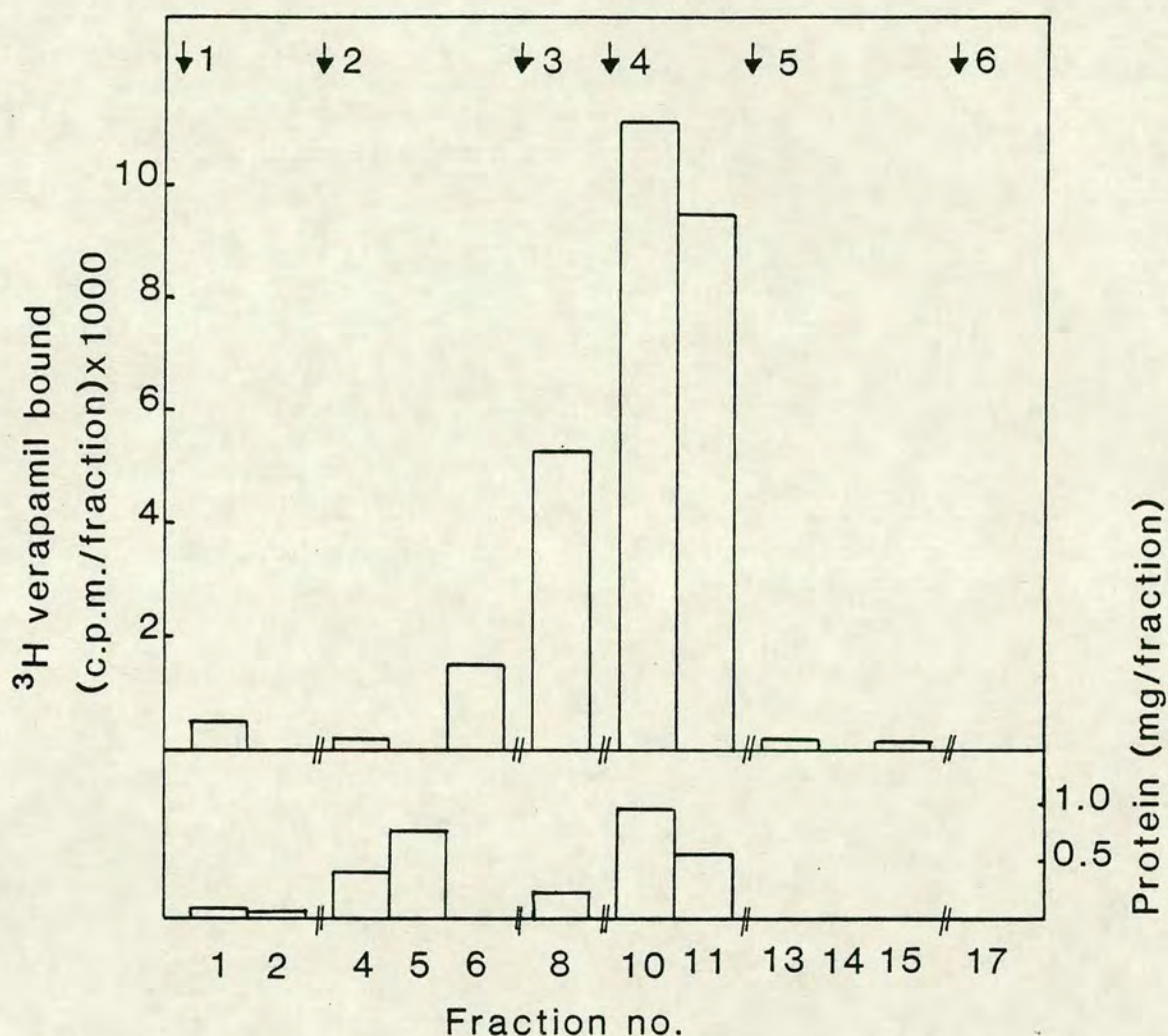


Fig.4.10. Phenyl Sepharose column elution profile using protocol 2.

Approx. 4 ml of pooled peak verapamil binding fractions from the DEAE column were adjusted to 1 M $(\text{NH}_4)_2\text{SO}_4$ and 1 mM CaCl_2 and loaded onto a 4 x 1 cm phenyl Sepharose column which had been pre-equilibrated high salt buffer. The column was then washed with high salt buffer (1), followed by low salt buffer (2), low Ca^{2+} buffer (3), minus Tris/Mops buffer (4), minus Ca^{2+} buffer (5) and d. H_2O (6). Fractions of 4 ml were collected and assayed for [^3H] verapamil binding. An A_{280} trace was used to indicate protein elution from the column. Fractions were collected when the trace deviated from a basal level. Fractions containing no protein were not collected and are indicated by // on the graph.

(Fig.4.10., Table 4.3.). The main conclusion drawn from the use of this protocol was that the presence of high salt improves the loading of the sample.

In further investigations into the optimum loading, washing and elution conditions, the effects of low detergent & sucrose concentration, were examined.

(Protocol 3., Fig.4.11.). In summary the sample was loaded in high salt followed by elution with low salt, minus Tris/Mops buffer (to give low detergent/sucrose only) and minus Tris/Mops buffer (to give high detergent sucrose only). With this protocol 83% of the activity initially loaded onto the column was recovered. 50% of this activity with relatively high specific activity was eluted immediately in the load and wash step. (Fig.4.11., Table.4.3.). Low salt led to the elution of a very small amount of binding with low specific activity. The remaining activity was eluted with low detergent/sucrose only and high detergent/sucrose only elution buffer. The latter elution step had the highest specific activity. (Table.4.3.). The conclusions from this protocol were that loading the sample in low detergent/sucrose does not promote the binding of the sample to the column matrix and minus Tris/Mops (low detergent/sucrose) elution followed by minus Tris/Mops (high detergent/sucrose) elution gives good recovery of the verapamil binding protein with high specific activity.

As a result protocol 4 used high salt, high detergent/sucrose loading conditions, followed by elution

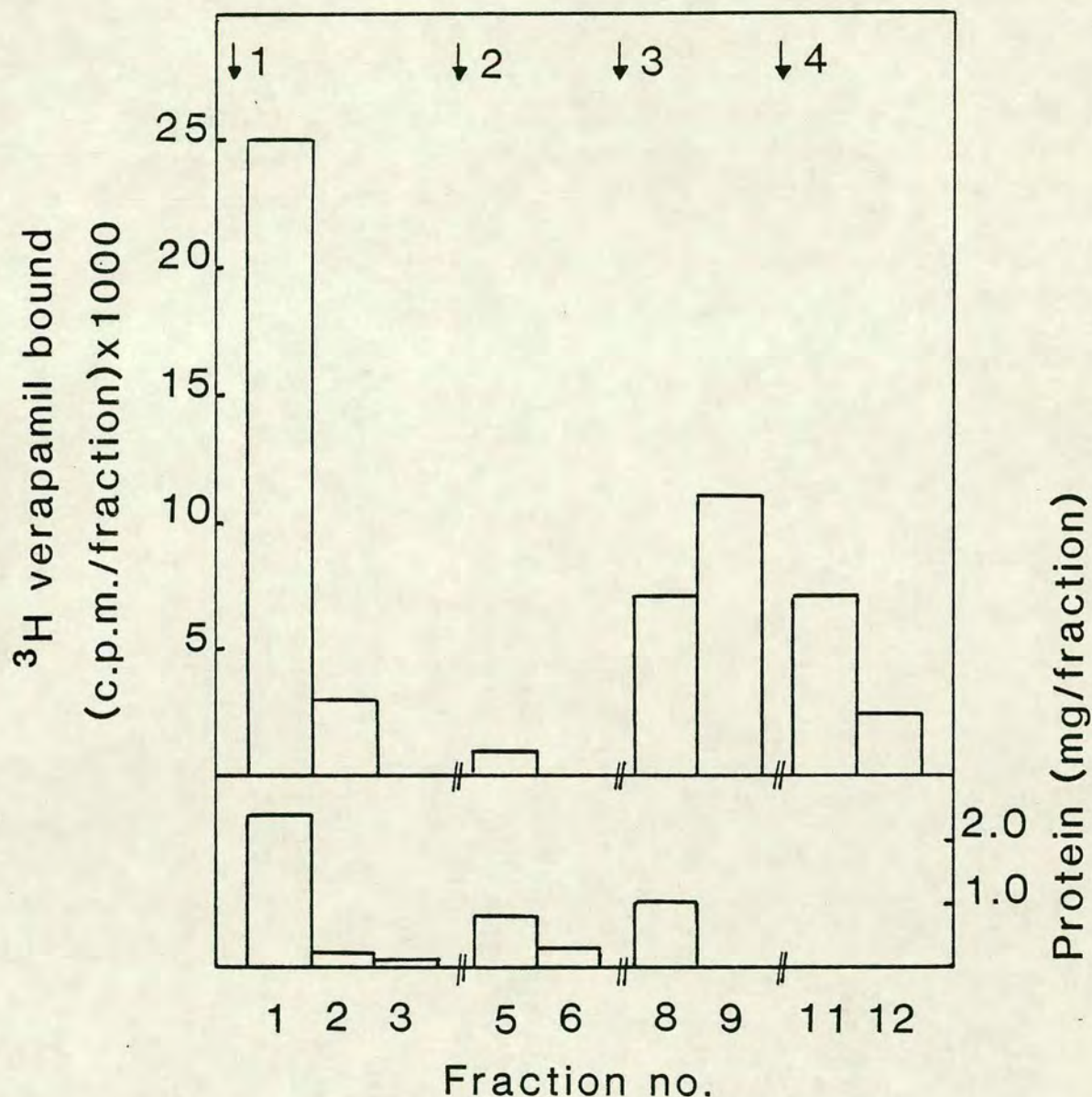


Fig.4.11. Phenyl Sepharose column elution profile using protocol 3.

Approx. 4 ml of pooled peak verapamil binding fractions from the DEAE column were adjusted to 1 M $(\text{NH}_4)_2\text{SO}_4$ and 1 mM CaCl_2 and loaded onto a 4 x 1 cm phenyl Sepharose column which had been pre-equilibrated with low detergent/sucrose buffer. The column was then washed with the same buffer (1), followed by low salt buffer (2), minus Tris/Mops (low detergent/sucrose) buffer (3) and minus Tris/Mops (high detergent/sucrose) buffer (4). Fractions of 4 ml were collected and assayed for [^3H] verapamil binding.

An A_{280} trace was used to indicate protein elution from the column. Fractions were collected when the trace deviated from a basal level. Fractions containing no protein were not collected and are indicated by // on the graph.

with low salt (which should elute a high proportion of the protein according to previous protocols), minus Tris/Mops (low detergent/sucrose) and minus Tris/Mops (high detergent/sucrose). (Fig.4.12.). 35% of the activity initially loaded was recovered using this protocol. The graph indicates that the sample loaded successfully onto the column, with only 14% of the total activity recovered lost at this stage. Low salt led to the elution of a large proportion of the protein (Fig.4.12.), and a small amount of activity (Table.4.3.). Elution with minus Tris/Mops (low detergent/sucrose), included to cause the elution of a relatively large amount of verapamil binding activity with high specific activity, failed to do so in this case; a small amount of binding activity with low specific activity was eluted from the column. However, use of minus Tris/Mops (high detergent/sucrose) resulted in the elution of the majority of the binding activity with high specific activity. (Table.4.3.).

From these investigations the following conclusions were drawn.

1. Successful loading of the verapamil binding activity onto the column requires the presence of high salt and a high concentration of detergent & sucrose.
2. If the sample is loaded under these conditions, most of the protein is eluted by decreasing the salt concentration.

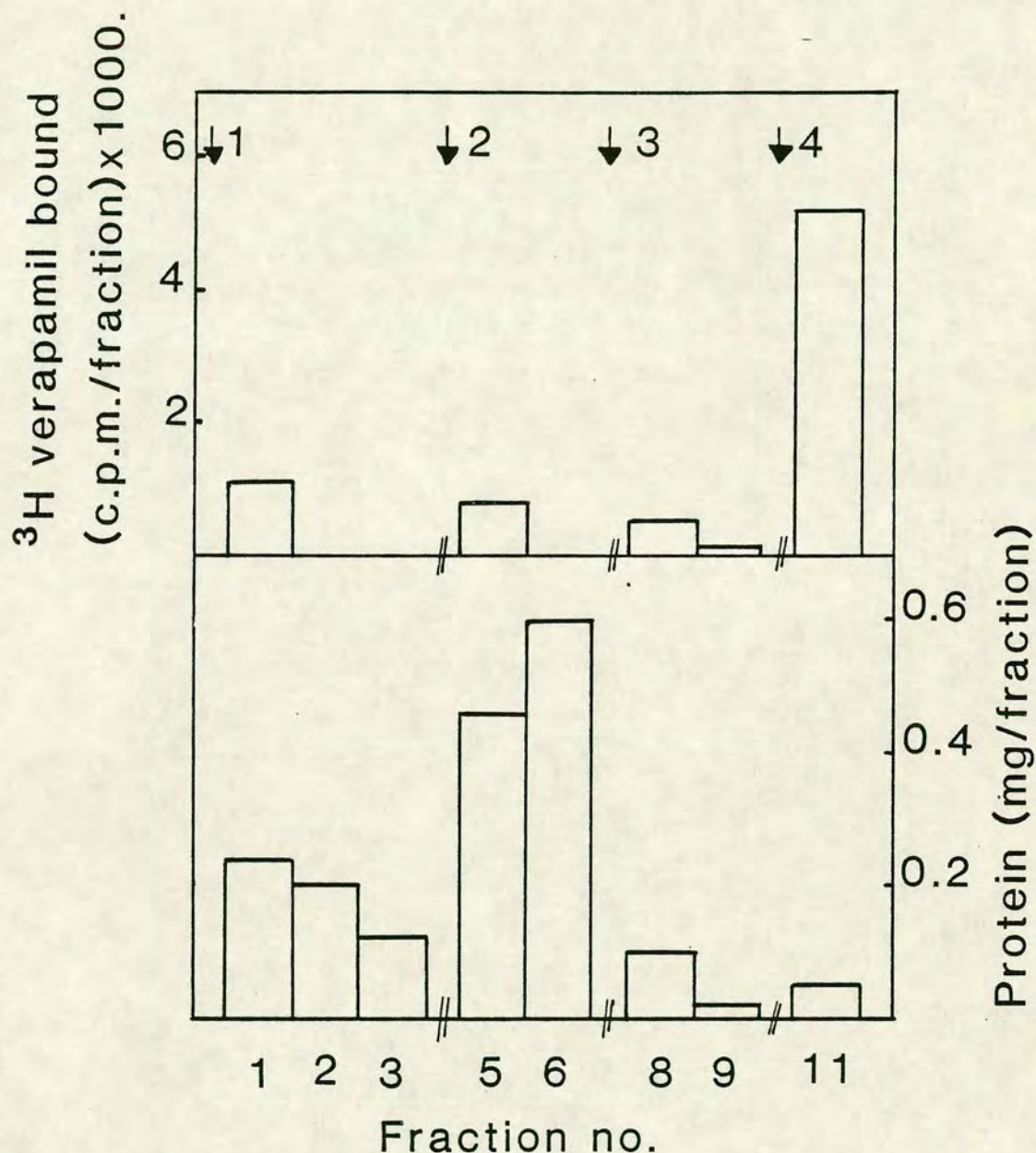


Fig.4.12. Phenyl Sepharose column elution profile using protocol 4.

Approx. 4ml of pooled peak verapamil binding fractions from the DEAE column were adjusted to 1 M $(\text{NH}_4)_2\text{SO}_4$ and 1 mM CaCl_2 and loaded onto a 4 x 1 cm phenyl Sepharose column which had been pre-equilibrated with high salt buffer. The column was then washed with the same buffer (1), followed by low salt buffer (2), minus Tris/Mops (low detergent/sucrose) buffer (3) and minus Tris/Mops (high detergent/sucrose) buffer (4). Fractions of 4 ml were collected and assayed for [³H] verapamil binding. An A_{280} trace was used to indicate protein elution from the column. Fractions were collected when the trace deviated from a basal level. Fractions containing no protein were not collected and are indicated by // on the graph.

3. A small but significant amount of verapamil binding protein with high specific activity is eluted by lowering the calcium concentration in the elution buffer.

4. The majority of the binding protein with high specific activity is eluted upon removal of the buffer component of the elution step i.e. with high detergent/sucrose only.

These conclusions led to the design of the final elution protocol which was, loading and washing in high salt buffer followed by sequential elution with low salt, low Ca^{2+} and high detergent/sucrose only.

4.5.2. The final elution protocol.

Fig.4.13. illustrates a typical phenyl Sepharose elution profile using the final protocol to separate pooled S400 fractions containing peak verapamil activity. Total recovery of binding activity from the column was 46% of that originally loaded. During loading and washing a small amount of binding with low specific activity was eluted from the column. The lowering of salt and calcium led to the elution of 39% and 10% of the binding activity respectively with relatively high specific activity. Elution with high detergent/sucrose only led to the elution of 34% of the total binding activity with the highest specific activity. (Table.4.3.).

Data from eight experiments utilising the final elution protocol indicated that the average fold purification of the verapamil binding protein after

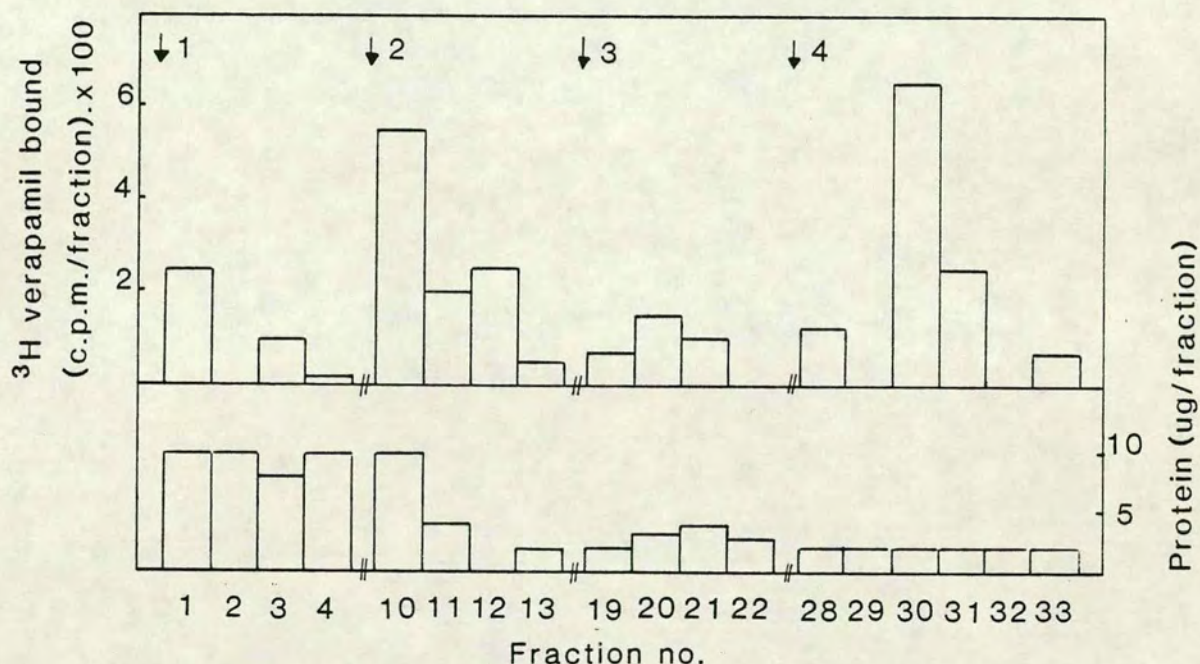


Fig.4.13 Phenyl Sepharose column elution profile using the final elution protocol (protocol 5). Approx. 4ml of pooled peak verapamil binding fractions from the Sephacryl S400 column were adjusted to 1 M $(\text{NH}_4)_2\text{SO}_4$ and 1 mM CaCl_2 and loaded onto a 2 x 1 cm phenyl Sepharose column which had been pre-equilibrated with high salt buffer. The column was then washed with upto 18 ml of high salt buffer and then eluted with 18 ml each of low salt buffer, low Ca^{2+} buffer and minus Tris/Mops buffer. Fractions of 2ml were collected and the first four 2 ml fractions of each elution step were assayed for [^3H] verapamil binding.

phenyl sepharose fractionation of the S400 fraction, was 4 ± 1.58 (n=8). Total fold purification i.e. after all the purification steps, was 39 ± 15.6 (n=8) and recovery of protein was $0.27 \pm 0.09\%$ (n=8) of that in the initial crude membrane preparation. This was equivalent to an average of 0.14 ± 0.019 (n=8) mg of protein found in the peak phenyl Sepharose fraction. The figures have been adjusted for losses in activity incurred earlier in the purification. Losses in binding activity during the phenyl Sepharose column run were negligible.

4.6. Lectin Affinity Column Chromatography.

Lectin affinity chromatography, which purifies glycoproteins, was used in the purification of the skeletal muscle calcium channel by taking advantage of the glycoprotein nature of the channel. Following this precedent set in the animal system, the purification of the verapamil binding protein from solubilised membrane fractions and DEAE fractions, was also attempted using both wheat germ lectin (coupled to agarose) and Con-A Sepharose.

This section outlines some of the approaches taken to purify the verapamil binding protein and to characterise its possible sugar specific residues using lectin affinity chromatography.

Solubilised membrane fractions were applied to Con-A Sepharose and wheat germ lectin (WGA). Fig.4.14. and

Fig.4.15. illustrate the elution profiles for the Con-A and wheat germ lectin columns respectively.

Using the Con-A Sepharose column, 61% of the binding activity initially loaded onto the column was recovered. Of the total activity recovered from the column, 72% was eluted in the load supernatant, together with the major protein peak (fraction 1.). In the subsequent wash step a further 18% of the total recoverable activity was eluted from the column (fraction 2.). Elution of the column with 0.1 M, 0.3 M and 0.4 M α -methyl mannoside resulted in no recovery of activity (fraction 1,3,4.). 0.2 M and 0.5 M α -methyl mannoside eluted 6% and 3% of the verapamil binding activity from the column respectively (fraction 2,5.). No purification of the verapamil binding protein was achieved using Con-A sepharose. This result suggests that the verapamil binding protein does not have the specific sugar residues (α -D-mannopyranosyl, α -D-glucopyranosyl and sterically related residues), to interact with Con-A lectin.

Using the wheat germ lectin column, 88% of the binding activity initially loaded onto the column was recovered. 81% of the total activity recovered from the column was eluted in the load SN and was eluted with the major protein peak (fraction 1.). 10% of total binding activity was eluted from the column in the following wash step (fraction 2.) and approximately 3% of total binding activity was eluted with 100 mM n-acetyl-glucosamine (fraction 3). Again purification of the verapamil binding

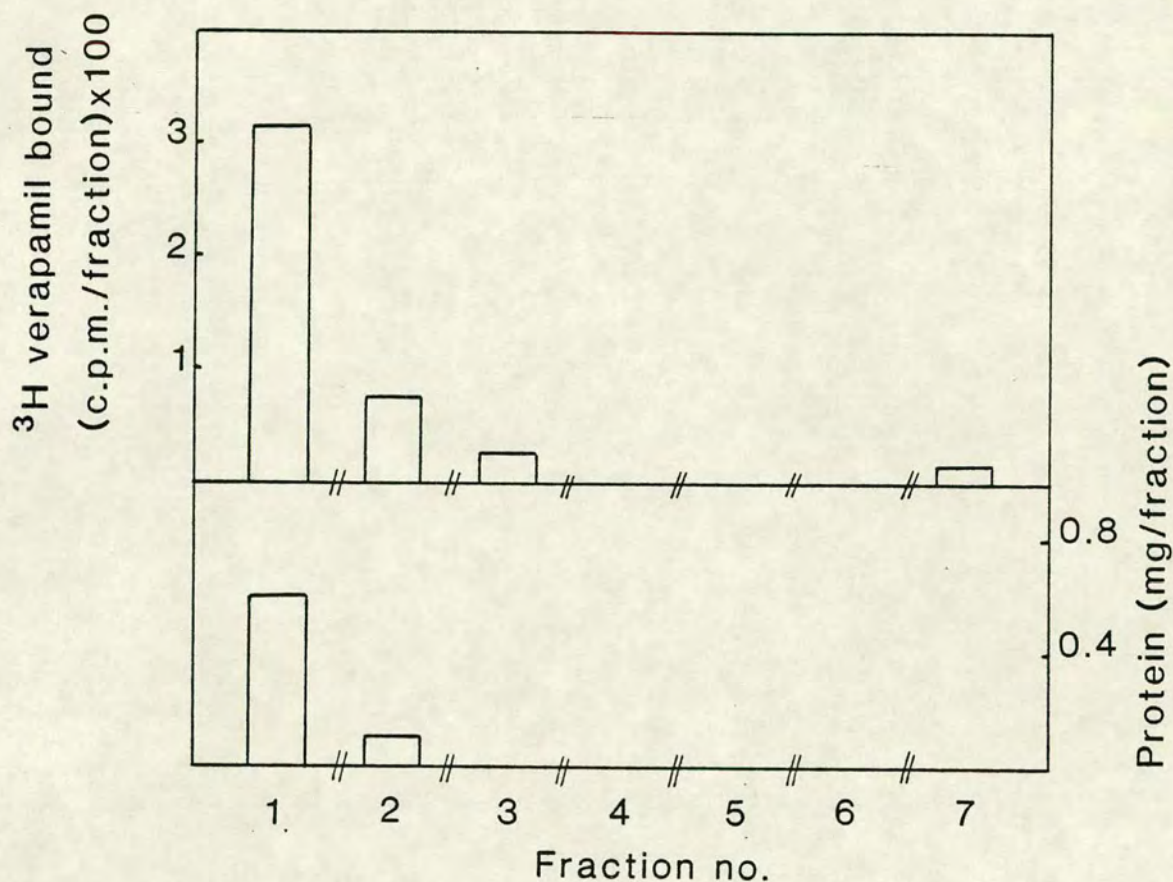


Fig.4.14. Con-A Sepharose elution profile.

Approx. 1 ml of solubilised membrane fraction was applied to the Con-A Sepharose which had been washed with Con-A column buffer. The Con-A plus sample were incubated for 2 h at 4°C and then centrifuged. The supernatant, referred to as the load supernatant (LSN) was removed and assayed for [^3H] verapamil binding (fraction 1). The Con-A was washed with Con-A column buffer and centrifuged. The wash supernatants (WSN) were pooled and assayed for [^3H] verapamil binding (fraction 2). The Con-A was then sequentially eluted with 1 ml each of 0.1 M, 0.2 M, 0.3 M, 0.4 M, 0.5 M α -methyl mannoside in Con-A column buffer. The supernatants from each elution step (ESN) were assayed for [^3H] verapamil binding (fraction 3-7).

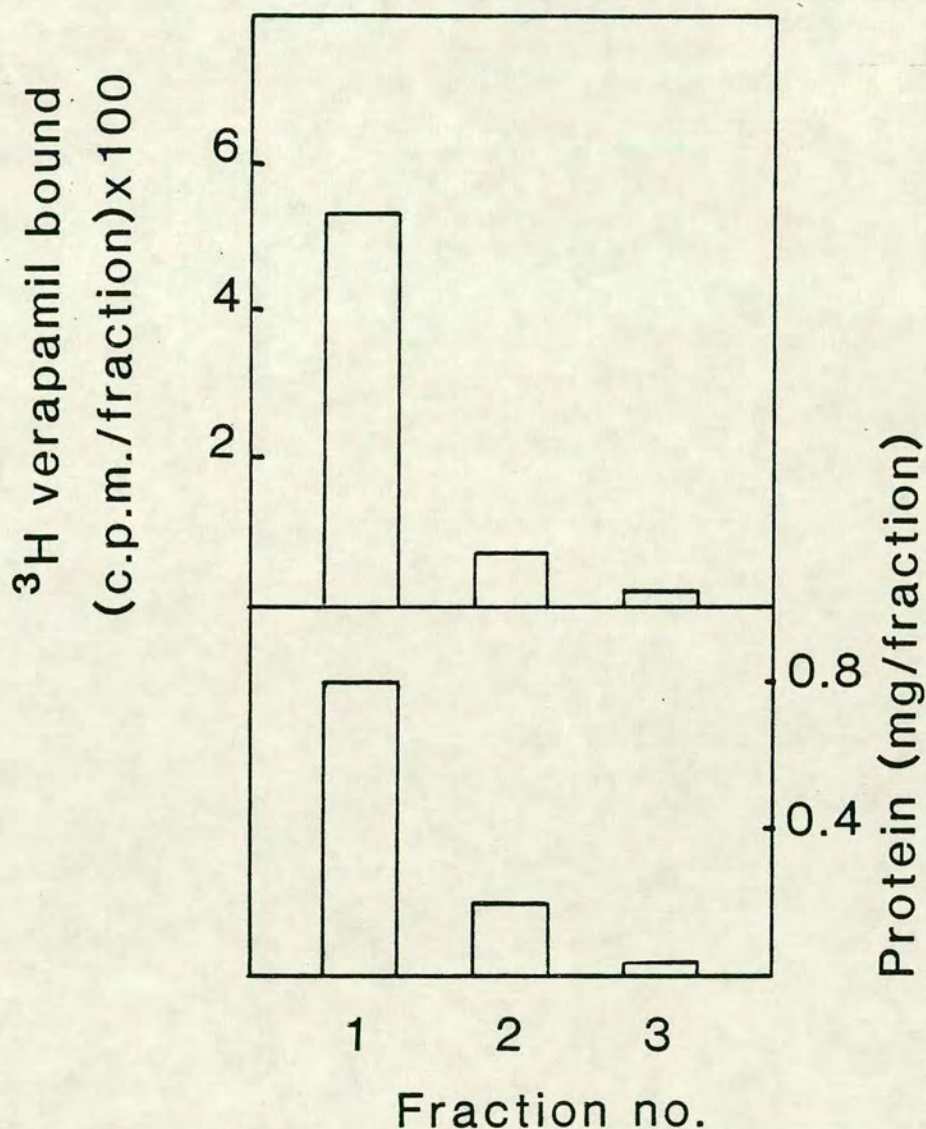


Fig.4.15. Wheat Germ Agarose column elution profile. Approx. 1 ml of solubilised membrane fraction was applied to the Wheat Germ Agarose which had been washed in column buffer. The WGA plus sample were incubated for 2 h at 4°C and then centrifuged. The WGA was washed and then eluted with 100 mM n-acetyl-D-glucosamine in column buffer. The load supernatant (fraction 1), wash supernatant (fraction 2) and elution supernatant (fraction 3) were assayed for [^3H] verapamil binding.

protein was not achieved with wheat germ lectin. The results suggest that the verapamil binding protein does not have the specific sugar residues (N-acetylglucosaminyl residues), to interact with wheat germ lectin.

With both Con-A and WGA, incubation of the protein sample with the matrix for up to 24 hours at 4⁰C, did not alter the elution behaviour of the verapamil binding activity, i.e. the major part of the verapamil binding activity eluted in the load SN.

Approximately 60% and 80% of the activity initially loaded was recovered from the Con-A and WGA columns respectively. This suggests that a proportion of the loaded activity remains bound to the column and/or the binding protein may have lost its activity during the experiment.

Using WGA and Con-A, the greater part of the verapamil binding activity recovered in total from the lectin columns was eluted from the column immediately (during loading of the sample and the subsequent wash steps). This indicates that the verapamil binding protein may not be a glycoprotein or has sugar residues which do not interact with Con-A or WGA. Other lectin types need to be tested to confirm this. Alternatively, the verapamil binding protein may be a glycoprotein but during the earlier stages of purification the appropriate sugar residues, which would allow interaction of the protein with the lectin, have been removed.

As the purification achieved was negligible and because verapamil binding activity was not retained by Con-A or WGA, lectin affinity chromatography was not used in the purification of the verapamil binding protein.

4.7. SDS Polyacrylamide Gel Electrophoresis.

After each purification step, (solubilisation, DEAE, gel filtration and phenyl Sepharose columns), the proteins contained in the different fractions were analysed by gel electrophoresis under reducing conditions.

Fig.4.16. shows a 10% SDS polyacrylamide gel of the purification steps. The gel has been stained with Coomassie blue, followed by silver staining. Lane 1 shows the protein content of the solubilised CHAPS extract. The heavy staining of this lane indicates the presence of many proteins.

After fractionation of the solubilised extract on a DEAE column, the DEAE fractions with peak verapamil binding activity still have many other proteins associated with the verapamil binding protein, (lane 2). The slightly less dense staining of the lane suggests that a small proportion of the associated proteins have been removed by this step, which corresponds to the low fold purification (average 2 fold), achieved after DEAE fractionation of the solubilised sample.

Lane 3 indicates the protein content of the S400 fraction containing peak verapamil binding activity.

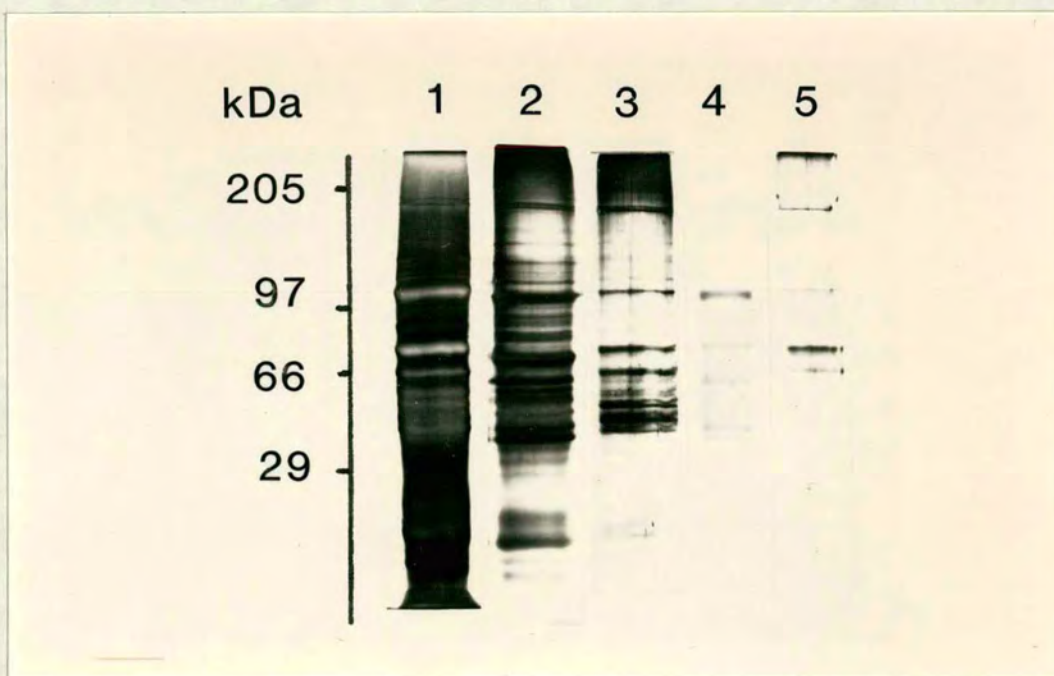


Fig.4.16. SDS PAGE of [^3H] verapamil binding fractions from maize coleoptile membranes at different stages of purification.

Lane 1, CHAPS solubilised membrane extract (5 μg of protein); Lane 2, pooled peak verapamil binding DEAE fraction (5 μg of protein); Lane 3, pooled peak verapamil binding Sephacryl S400 fraction (5 μg of protein); Lane 4, Sephacryl S400 fraction with no verapamil binding activity (5 μg of protein); Lane 5, minus Tris/Mops (high detergent/sucrose) fraction from the phenyl Sepharose column (1 μg of protein). The proteins were visualised by staining the gel with Coomassie blue and silver.

Substantially more of the associated proteins have been removed after this step, which reflects the average purification of approx. 13 fold found after fractionation of S400 fractionation of the DEAE sample.

Lane 4 shows the protein content of an S400 major protein fraction containing no verapamil binding activity for comparison. It is noticeable that the band of Mr 169,000 is absent in the protein profile of this fraction, together with some bands of lower molecular weight.

The final purification step, lane 5, contains four stained bands of Mr 169,000, Mr 100,000, Mr 70,000 and Mr 66,000. The large reduction in the number of proteins present in the peak binding fraction after the final purification step, suggests that there has been extensive purification of the verapamil binding protein. The bands of Mr 169,000 and 70,000 appear to become enriched during the purification and their enrichment follows the increase of specific binding activity of [^3H] verapamil. The band of Mr 169,000 was found to be absent from the protein profiles of inactive fractions (lane 4), but present in active fractions. This suggests that this protein may be essential for the binding of verapamil. Indeed, it may be the verapamil binding protein/receptor and be part of a plant calcium channel. Could any of the other protein bands in the final fraction be the verapamil binding protein? The band of Mr 100,000 is considered to be a contaminant protein, as this protein

was observed in both verapamil binding and non-verapamil binding fractions from the Sephacryl S400 column and the breakthrough fractions from the DEAE column. The protein of Mr 70,000 may also be a contaminant, as a protein of equivalent molecular weight was observed in some of the non-verapamil binding fractions of the Sephacryl S400 elution profile. The band of Mr 66,000 is also discounted as being the verapamil binding protein or part of a calcium channel, as this band is a staining artefact from keratin, frequently observed even in unloaded gel slots and reported by others (Ochs, 1983).

On the basis of these observations, the protein of Mr 169,000 is suggested to contain the verapamil binding site. It is also possible that the Mr 70,000 band may bind verapamil or perhaps be an associated channel protein. The band of Mr 169,000 is thought to be the more likely candidate for the verapamil binding protein, as this polypeptide is comparable in size to the α_1 subunit of the L-type calcium channel from skeletal muscle, which has a molecular weight of 155-200 kDa (Glossmann & Streissnig, 1988) and contains the receptor sites for dihydropyridines and phenylalkylamines (Striessnig et al, 1986,1987).

There are no comparable calcium channel subunits in skeletal muscle, of molecular weight similar to the band of Mr 70,000 observed here. However, on this basis alone, the Mr 70,000 band cannot be discounted as a channel

subunit, as plant calcium channels may have different sized subunits compared to their animal counterparts.

4.8. The Purification Procedure: Summary and Discussion.

The putative verapamil binding protein was partially purified using a combination of detergent solubilisation, ion exchange, gel filtration and hydrophobic interaction column chromatography. In summary the purification protocol was as follows. The membrane bound verapamil binding protein was solubilised using 0.5% CHAPS and 20% sucrose in buffer at a protein detergent ratio of 1:14 (w/w). The solubilised verapamil binding protein was then subjected to DEAE ion exchange chromatography and the verapamil binding protein eluted from the column with 0.15 M NaCl in column buffer. Fractions containing verapamil binding activity were further fractionated on a Sephacryl S400 column. Peak verapamil binding fractions, eluting in the void volume of this column, were then applied to a phenyl Sepharose column in high salt buffer. The column was eluted with low salt, low Ca^{2+} and minus Tris/Mops elution buffer, with the verapamil binding protein being eluted in the Tris/Mops elution buffer. The proteins from the final purified fraction were then separated by gel electrophoresis. This revealed four stained bands, one of which (M_r 169,000) was proposed to be the putative verapamil binding protein.

The purification protocol used in the research presented in this thesis, can be compared to that

employed to purify the dihydropyridine-sensitive, voltage-dependent (L-type) calcium channel from skeletal muscle. One of the main differences in the methodology adopted to purify the verapamil binding protein and that used to purify the dihydropyridine receptor, was the absence of lectin affinity chromatography in the final verapamil binding protein purification protocol. Lectin affinity chromatography was very successful in the purification of the dihydropyridine receptor, because although the receptor subunit (α_1) was not itself a glycoprotein, it was associated with glycosylated channel proteins and so could be purified. (Using wheat germ lectin the dihydropyridine receptor/skeletal muscle calcium channel was purified 38.2 fold (Borsotto et al, 1985)). The use of lectins was attempted, but the verapamil binding protein in solubilised maize coleoptile membrane preparations and DEAE fractions did not adhere successfully to Con-A Sepharose or Wheat germ lectin columns. This suggested that the verapamil binding protein was not a glycoprotein. If the putative verapamil binding protein is directly comparable to the α_1 subunit of the skeletal muscle calcium channel, this might be expected. As no purification of the verapamil binding protein was achieved using WGA or Con-A Sepharose, lectin affinity chromatography was not employed in the purification protocol.

Table 4.4. summarises the average purification data obtained using the final purification protocol. It is

PURIFICATION STEP.	RECOVERY OF ACTIVITY.			PROTEIN.			SPECIFIC ACTIVITY.	FOLD PURIFICATION.	
	C.P.M.	% STEP	% TOTAL.	mg.	% STEP.	% TOTAL.		STEP.	TOTAL.
CRUDE MEMBRANE.	226853 ± 64618	100	100	45 ± 5.5	100	100	5793 ± 1625	-	-
CHAPS EXTRACT.	824070 ± 244426	297 ± 58.5	297 ± 58.5	57 ± 14.6	119 ± 19.2	119 ± 19.2	19242 ± 6295	2.62 ± 0.53	2.62 ± 0.53
DEAE.	113954 ± 31165	69.3 ± 41.7	111 ± 52.6	14.5 ± 2.3	30.9 ± 5.6	30.6 ± 6.0	9940 ± 3510	2.25 ± 1.28	3.37 ± 1.41
S400.	59040 ± 17569	106 ± 48.0	74.2 ± 39.6	2.5 ± 1.0	16.1 ± 4.7	5.0 ± 1.4	51291 ± 15925	13.5 ± 8.17	22.5 ± 9.59
PHENYL SEPHAROSE.	6228 ± 2156	21.2 ± 15.6	6.6 ± 3.7	0.09 ± 0.03	5.97 ± 1.28	0.2 ± 0.06	94053 ± 19568	3.95 ± 1.58	36.3 ± 16.2

Table 4.4. Averaged values for the recovery of binding activity, recovery of protein, specific activity and fold purification obtained using the final purification protocol.

Values shown for crude membrane, CHAPS extract, DEAE and S400 are ± SE (n=9). Values for phenyl Sepharose are ± SE (n=7).

noticeable that there is variation in the degree of purification of the verapamil binding protein within a single stage of the procedure. The reasons for this variation probably lies in differences in the specific activity of the starting material and the degree of purification obtained in the previous purification steps. The activity of the crude membrane may vary according to how well the tissue was homogenised. Up to 50% of the plasma membrane can be lost with the cell walls during homogenisation and centrifugation (Hall, 1983). Therefore if the homogenisation procedure is not totally uniform between experiments, the amount of activity will vary between preparations. Activity of the solubilised verapamil binding protein may also vary because although the tissue is solubilised at a strict protein:detergent ratio, variation in activity may occur if the preceeding homogenisation step has resulted in more or less membrane protein being available for solubilisation.

Data from a number of ion exchange separations of the solubilised membrane fraction, indicated that where solubilisation of the crude membrane extract resulted in a significant purification of verapamil binding activity, the fold purification after DEAE column chromatography tended to be very low. This is probably because the solubilisation and DEAE steps are removing similar proteins, so that if most of these non-verapamil binding proteins are removed during solubilisation of the crude

membrane preparation, very few will be left to be removed by the DEAE column, thus resulting in low purification after ion exchange. Similarly where solubilisation of the crude membrane fraction has been poor, resulting in low purification after this step, the fold purification after the DEAE column tended to be higher. Therefore depending on the specific activity of the crude membrane and degree of purification of verapamil binding activity during solubilisation, the overall purification of the verapamil binding protein after ion exchange will vary between experiments. The average fold purification values obtained after solubilisation of the membrane bound receptor and DEAE fractionation of the solubilised verapamil binding protein were 2.62 ± 0.53 fold (n=9) and 2.25 ± 1.28 fold (n=9) respectively.

S400 fractionation of DEAE fractions containing peak verapamil binding activity also gave variable amounts of purification depending on the success of the previous steps. However the S400 column consistently resulted in the highest purification of the verapamil binding protein compared to the other steps of the protocol. On average 13.5 ± 8.1 fold (n=9) purification was achieved with this step. Separation of the S400 fraction on a phenyl Sepharose column resulted in an average 3.96 ± 1.58 fold (n=7) purification of the verapamil binding protein.

It therefore appears that the ^Csuccess of all the steps of the purification procedure are dependent on each other and all of them appear to be necessary for the

partial purification of the verapamil binding protein, despite the fact that the level of purification achieved maybe low after individual columns.

Table 4.4. also indicates that there is variation in the recovery of binding activity within a single stage of the protocol. The figures have been adjusted for losses in activity due to freezing of the sample during the purification and decay of activity over time. This means that the observed variation is due to factors other than denaturation of the binding activity by freezing and over time. One explanation may be that some of the binding activity cannot be eluted and is therefore lost on the column.

Loss of binding activity over the duration of the purification protocol occurred mainly at the DEAE stage of the purification procedure. On average this loss was $42.5 \pm 15\%$ ($n=7$). The observed variation in the decay of activity is probably a reflection of the degree of purification achieved during solubilisation eg: if purification after solubilisation has been poor, more proteases may be present in the sample than if the solubilisation had resulted in a better purification. This may lead to increased loss in binding activity due to protease action. Denaturation of the verapamil binding protein does occur during purification, despite attempts to stabilise the protein by the inclusion of 20% sucrose in all the column buffers. In a similar fashion, exogenous lipid or glycerol were used to limit receptor

denaturation in the purification of the dihydropyridine receptor from skeletal muscle.

Using the final purification procedure, the average total purification i.e. after all the columns, of the verapamil binding protein was 36.3 ± 16.2 fold ($n=7$). In one experiment the total purification was as great as 121 fold. (Table.4.5.a.). However this figure is probably an over estimate due to the inaccuracies involved in measuring the very low levels of protein remaining in the final phenyl Sepharose fraction. The average purification figures quoted are probably more representative of the purifications that can actually be achieved using the final protocol. (Table.4.4. & Table.4.5.b.).

From SDS gel electrophoresis of fractions from the final purification step, a protein of Mr 169,000 is proposed as the putative verapamil binding protein. The gels indicate only one possible associated subunit, not four subunits as seen in gels of purified calcium channels from skeletal muscle (run under reducing conditions). This suggests either that this method may not purify the whole channel, or that the structure of the plant calcium channel is different compared to animals. No absolute identification of the stained bands has been made, although attempts to do so using photoaffinity labelling and [^3H] verapamil binding to western blots of purified fractions were made. These attempts are described later in this thesis. To check whether the Mr 169,000 is the calcium channel or part of

PURIFICATION STEP.	RECOVERY OF ACTIVITY.		PROTEIN.		SPECIFIC ACTIVITY.	FOLD PURIFICATION.
	C.P.M.	%	mg.	%		
CRUDE MEMBRANE.	73600	100	56	100	1302	
CHAPS EXTRACT.	32240	44	25	44	1305	1
DEAE.	10304	14	4	7	2576	2
S400	23800	32	0.24	0.42	99166	76
PHENYL SEPHAROSE.	1264	1.7	0.008	0.014	158000	121

Table 4.5.a. Values for the best purification achieved using the final purification protocol.

PURIFICATION STEP.	RECOVERY OF ACTIVITY.		PROTEIN.		SPECIFIC	FOLD PURIFICATION.
	C.P.M.	%	mg.	%	ACTIVITY.	
CRUDE MEMBRANE.	102853	100	13	100	7639	
CHAPS EXTRACT.	345032	335	15	115	22288	3
DEAE	58580	57	2.6	20	21858	3
S400	37028	36	0.32	2.3	115712	15
PHENYL SEPHAROSE.	2285	2.2	0.016	0.11	142750	18.68

Table 4.5.b. Typical purification values obtained using the final purification protocol.

the channel, would require the protein to be reconstituted into lipid bilayers and tested for channel activity by patch clamping, which was outside the scope of this thesis.

CHAPTER 5.

IDENTIFICATION AND VISUALISATION OF THE VERAPAMIL BINDING PROTEIN FROM MAIZE COLEOPTILES.

5.1. Introduction.

Two methods were employed to visualise a verapamil binding protein (or possibly proteins) in maize coleoptile membrane preparations and to verify whether the proposed protein of 169 kDa is the verapamil binding protein in plant membranes. 1. Photoaffinity labelling of the membrane bound and partially purified putative verapamil binding protein using the tritiated arylazido phenylalkylamine [^3H] LU49888, an azido analog of D888. 2. [^3H] verapamil binding to western blots of crude membrane fractions and to fractions after each stage of purification.

The results of these investigations are shown in the following section.

5.2. Photoaffinity labelling using [N-methyl- ^3H] LU49888.

5.2.1. [N-methyl- ^3H] LU49888 binding in crude membrane preparations from maize coleoptiles.

In initial experiments utilising [^3H] LU49888, the binding characteristics of the drug to maize coleoptile membrane fractions were investigated. The binding assay used to detect [^3H] LU49888 binding in crude membrane fractions, was essentially the same as that used for [^3H] verapamil binding.

Fig.5.1. illustrates [^3H] LU 49888 binding in crude membrane fractions. The graph shows that there is a much higher amount of non-specific binding compared to specific binding at all the concentrations of [^3H] LU 49888 examined. This is in contrast to [^3H] verapamil binding where non-specific binding only makes up about 4% of the total binding, but similar to D888 binding experiments where non-specific binding was approximately 50% of total binding. It is possible that the higher element of non-specific binding encountered here results from the azido group present in [^3H] LU 49888, which may cause the drug to bind to proteins other than just specific phenylalkylamine binding proteins. Differences in the binding characteristics between [^3H] verapamil and [^3H] LU49888 may also be expected, as LU49888 is structurally slightly different. It should also be noted that the binding assay for LU 49888 has not been optimised for this particular drug, which may account for some of the differences observed.

5.2.2. Photoaffinity labelling of the phenylalkylamine binding protein(s) from maize coleoptile membrane preparations.

A variety of experiments were conducted in an attempt to identify the verapamil binding protein(s) after separation of crude, solubilised and partially

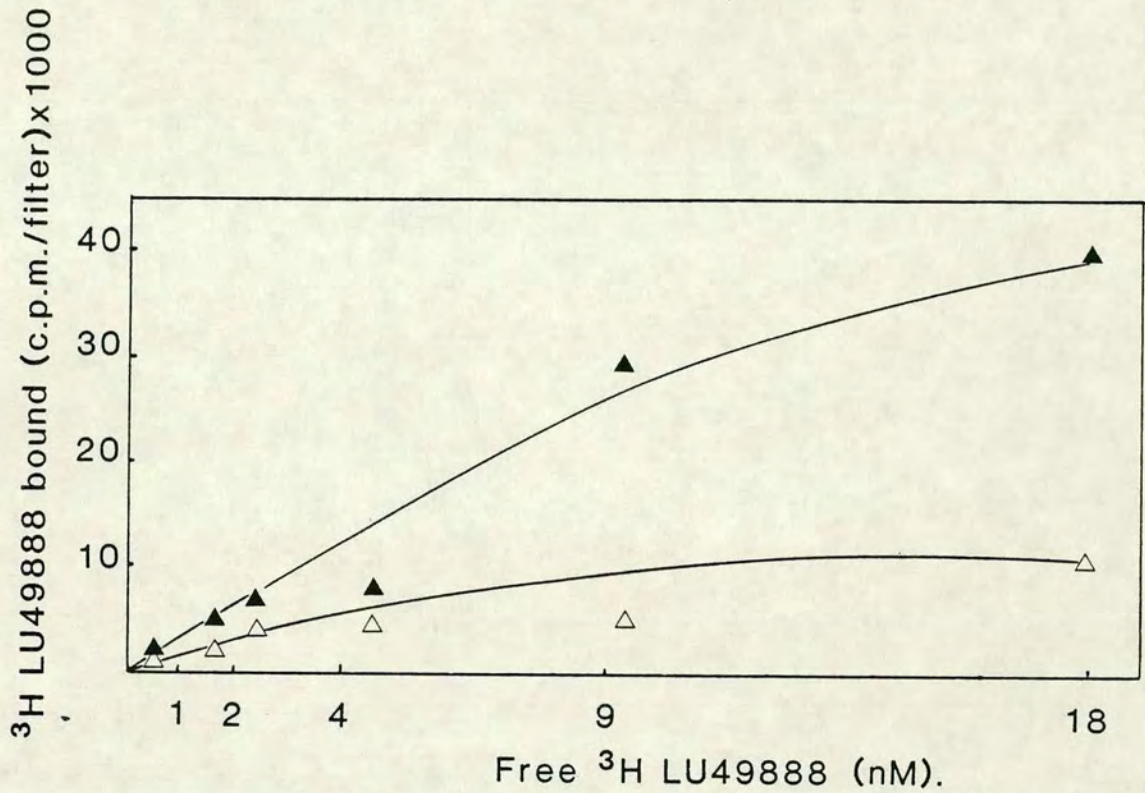


Fig.5.1. Binding of [^3H] LU 49888 to crude membrane fractions from maize coleoptiles.

[^3H] LU 49888 was incubated with crude membrane fractions (0.5 mg/500 μl assay) for 2 h at 20^0C in the dark, at the concentrations indicated (total binding). Non-specific binding (▲), was determined in parallel samples containing 100 μM unlabelled D888 and was subtracted from total binding to give specific binding (Δ).

purified membrane fractions by SDS PAGE. The methods employed were essentially the same as those used by Ferry *et al.* (1984b). In particular it was hoped to determine whether the 169 kDa protein seen on SDS polyacrylamide gels was the verapamil binding protein as proposed earlier in this thesis.

Membrane fractions were incubated with [^3H] LU 49888 and irradiated with UV light. This activates the azido group which forms a covalent link with specific proteins. The labelled samples were then separated by gel electrophoresis, fixed and stained with Coomassie blue. Initially the gel was incubated in EN 3 HANCE, dried down and then autoradiographed. However, no bound [^3H] LU 49888 was observed, possibly because inadequate quantities of bound radioactivity were loaded onto the gel initially, which prevented detection of the binding proteins. Tritium is also difficult to detect by ^{Fluorography?} autoradiography. Following the failure of this method, the dried down gel was then cut into slices and the radioactivity incorporated into each slice determined by scintillation counting, as this is more sensitive than autoradiography. Fig.5.2. & Fig.5.3. show labelling of proteins from crude membrane and DEAE fractions after separation by SDS PAGE, using this method.

Fig.5.2. indicates that a protein of apparent molecular weight 30 kDa is photolabelled with [^3H] LU 49888 in crude membrane fractions. No other proteins appear to be significantly labelled. Several



Fig.5.2. Photoaffinity labelling of crude maize coleoptile membrane fractions using [³H] LU 49888 (method 1).

Crude membrane fractions (0.5-1.0 mg/100-200 μ l) were incubated with a final concentration of 18 nM [³H] LU 49888 (total binding). Non-specific binding was determined by incubating membrane with [³H] LU 49888 plus 100 μ M of unlabelled D888. Specific binding (shown on this graph) was determined by subtracting non-specific binding from total binding. After incubation for 2 h at 20°C in the dark, the samples were photolabelled and separated by SDS PAGE. The gel lanes were cut into 2 mm slices, solubilised in alkali and the radioactivity incorporated into each slice determined by scintillation counting. The figure shows the results from the top of the gel (slice no.1). The arrows indicate the position of the molecular weight markers.

possibilities exist as to the identity of the labelled 30 kDa protein: It maybe a subunit of a plant membrane calcium channel, which can bind phenylalkylamines. Subunits of a similar size have been found to be part of the L-type calcium channel from skeletal muscle, but they do not contain drug binding sites; it may be a proteolytic fragment of the 169 kDa peptide, which has been labelled by the [^3H] LU 49888. However one may expect to see more fragments if this were the case; it may represent a low affinity phenylalkylamine binding site which is unrelated to the calcium channel drug binding sites. This type of binding has been identified in work with various animal tissues where [^3H] nitrendipine plus high intensity UV irradiation identified components with low Mr (20,000 to 50,000) (Campbell *et al.*, 1983; Horne *et al.*, 1984).

The same photoaffinity technique was applied to DEAE fractions. (Fig.5.3.). The graph indicates that the labelling is fairly widespread, making it difficult to identify any definite peaks of [^3H] LU 49888 incorporation. The area of high incorporation at the top of the gel is probably due to contamination from the stacking gel which tended to accumulate protein and label. It is possible that there may a peak of [^3H] LU 49888 incorporation corresponding to a protein of approx. molecular weight 48 kDa, but apart from this the incorporation of label is found to some extent in every gel slice. This suggests that the phenylalkylamine

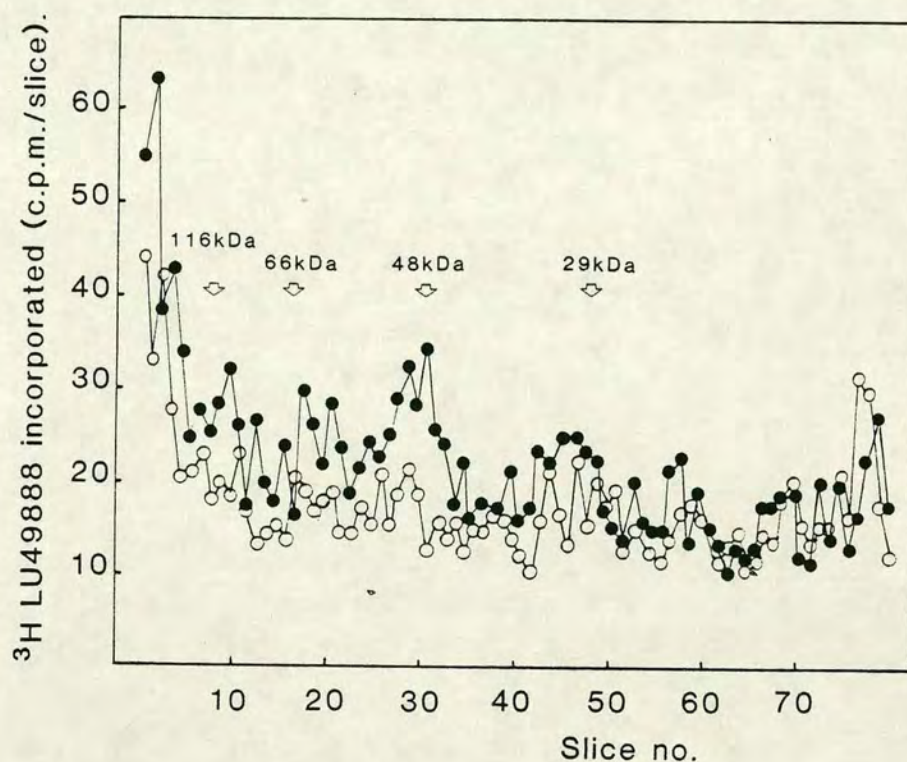


Fig.5.3. Photoaffinity labelling of DEAE fractions containing peak verapamil binding activity, using [^3H] LU 49888.

DEAE fractions (0.1-0.5 mg/100-200 μl) were incubated with a final concentration of 18 nM [^3H] LU 49888 (total binding ●). Non-specific binding was determined in samples containing [^3H] LU 49888 plus 100 μM unlabelled D888 (○). After incubation for 2 h at 20°C in the dark, the samples were photolabelled and separated by SDS PAGE. The gel lanes were cut into 2 mm slices, solubilised in alkali and the radioactivity incorporated into each slice determined by scintillation counting. The figure shows the results from the top of the gel (slice 1). The arrows indicate the position of the molecular weight markers.

binding protein has fragmented during the photolabelling process, as reported for [^3H] LU 49888 labelling of the skeletal muscle calcium channel (Striessnig et al., 1987), or that the label has bound to any protein it has come into contact with during irradiation. Fragmentation of the phenylalkylamine binding protein is possible as no protease inhibitors are present at any stage of the purification or during photolabelling. However in the results presented here it is difficult to identify definite proteolytic fragments, as the incorporation of label appears to occur throughout the sample with no obvious peaks of incorporation. This type of labelling was also observed when S400 fractions were photolabelled with [^3H] LU 49888.

To reduce the amount of non-specific binding by the [^3H] LU 49888 to the membrane fractions and allow clearer labelling of any phenylalkylamine binding proteins, irradiation was performed after filtration and washing of the sample. (Method 2.27.2.). [^3H] LU 49888 is a highly reactive species, which, once it is activated by UV light appears to bind to any proteins in its vicinity, regardless of specificity. By irradiating the proteins plus label on the filter rather than the protein plus label in solution, it was hoped to remove some of the non-specific binding element by the filtering process and nature of the assay. When the incorporation of label by the irradiated filters was determined by scintillation counting, the results indicated that non-specific binding

was reduced to approximately 40-50% of the total binding compared to 70-80% of total binding obtained when the sample plus label was irradiated and then filtered. Despite the apparent reduction in non-specific binding, this element was still a substantial part of the total [^3H] LU 49888 binding when the incorporation of radioactivity was determined in gel slices. The gel slices also indicated that there was no incorporation of label into specific polypeptides extracted from the filters. The high amount of non-specific binding plus the likely loss of labelled proteins during extraction (not all the labelled proteins will have been extracted from the filter), prevented the identification of any labelled polypeptides in the gel slices.

5.3. [^3H] verapamil binding to Western blots of crude membrane fractions and to fractions containing the partially purified putative verapamil binding protein.

An alternative strategy was adopted to detect verapamil binding proteins. After separation on SDS PAGE, the proteins in crude membrane fractions and partially purified verapamil binding fractions were then transferred to nitrocellulose paper and incubated in [^3H] verapamil. [^3H] verapamil binding to the separated proteins was detected by autoradiography of the nitrocellulose blot.

Three methods were used to investigate [^3H] verapamil binding to the separated protein fractions.

(Method 2.24). Transfer of the proteins was essentially the same in all three methods.

In the first method the proteins were transferred to nitrocellulose paper, washed with Tween 20 and incubated in [^3H] verapamil. The Tween 20 was present to site saturate the blot. The blot was then washed, air dried and sprayed with EN 3 HANCE prior to autoradiography. On development of the autoradiograph, no localisation of [^3H] verapamil binding was evident. Instead the label appeared to be found all over the blot, suggesting that the Tween 20 had not been successful in site saturating the blotted proteins, so allowing the verapamil to bind indiscriminately to any proteins present.

In an attempt to alleviate this indiscriminate binding of [^3H] verapamil to the blot, a second method was adopted in which the blot was incubated in 10^{-5} M nifedipine followed by [^3H] verapamil. Nifedipine was used because it was known from earlier experiments that verapamil is unable to displace nifedipine and that nifedipine does not appear to bind to the same site as verapamil. Because of this, nifedipine should be an effective agent to site saturate the blotted proteins. No localised bands of [^3H]verapamil binding were detected in any of the fractions (crude, solubilised, DEAE, S400 and phenyl Sepharose), although non-specific binding was reduced using this method.

In the two methods described above, binding of [^3H] verapamil to denatured proteins was measured. Because the

proteins were denatured and therefore conformationally changed, this was a possible reason for the proteins not binding [^3H] verapamil. In the third method, the proteins were first renatured by incubation of the gels in buffer containing 0.1% CHAPS prior to Western blotting. 0.1% CHAPS was present throughout the whole experiment to keep the proteins renatured. The renatured proteins were then transferred to nylon membranes, incubated in nifedipine and then [^3H] verapamil, as before. Autoradiographs of the nylon membranes indicated that again no binding of [^3H] verapamil to specific proteins had occurred, despite renaturation of the proteins.

5.4. Discussion.

In summary, the verapamil binding protein was not detected by either photoaffinity labelling of membrane fractions prior to SDS PAGE or by [^3H] verapamil overlay procedures on nitrocellulose blots. Thus no further evidence was found to support the proposal that the 169 kDa protein found in partially purified preparations is the verapamil/phenylalkylamine binding protein.

Futher work is needed to investigate photoaffinity labelling of phenylalkylamine binding proteins in maize coleoptile membrane preparations and in fractions containing the partially purified verapamil binding activity. The major problem with the photoaffinity labelling appears to be the amount of non-specific binding of [^3H] LU 49888 to the membrane and purified

fractions. The use of carrot microsomal membrane fractions might overcome this problem as they bind D888 with higher affinity than maize membranes and with greater distinction between specific binding and non-specific binding (Graziana *et al.*, 1987). As [^3H] LU 49888 is an azido analog of D888, use of the photoaffinity label may be more successful in carrot membranes.

[^3H] verapamil binding to western blots of SDS gel separated fractions also proved to be unsuccessful. Using this technique, no specific phenylalkylamine/verapamil binding proteins were detected. It is possible that verapamil may not bind tightly enough to the blotted proteins and the label is removed during the washing of the blot. [^3H] verapamil may also fail to bind to the blotted proteins because during purification components vital to the binding of verapamil have been removed.

CHAPTER 6.

THE PRELIMINARY CHARACTERISATION OF THE PUTATIVE VERAPAMIL BINDING PROTEIN.

6.1. Introduction.

The calcium channel has been successfully identified using [^3H] verapamil. However, photoaffinity labelling and [^3H] verapamil overlays failed to identify the verapamil binding subunit(s) of the channel. Other techniques were therefore used to further characterise the verapamil binding protein and associated components.

L-type calcium channels from skeletal muscle have three glycosylated subunits, α_2 , γ and δ (Glossmann & Streissnig, 1988). The α_1 subunit, which contains the drug receptor sites, has glycosylation sites but no attached carbohydrate. The β subunit is also not glycosylated. These calcium channels are also known to be regulated by phosphorylation. The 165 kDa polypeptide (α_1 subunit) of the L-Type channel has been reported to act as a substrate for cAMP-dependent and calcium calmodulin-dependent protein kinases and can undergo multiple phosphorylation at distinct sites by these enzymes (O'Callahan, 1988). As well as these characteristics, calcium ions are thought to pass through the calcium channel by binding to divalent cation binding sites within the channel pore (Hess & Tsien, 1984). From

studies of the amino acid sequence of the α_1 subunit of the L-Type calcium channel, regions resembling areas of calcium binding proteins have been detected and electrophysiological and biochemical data suggests that there are at least two divalent cation binding sites in the channel (Tanabe et al., 1987).

This chapter outlines the results from preliminary experiments carried out to investigate whether the verapamil binding protein and associated components from plant membranes are; 1. glycoprotein in nature. 2. regulated by phosphorylation. 3. calcium binding proteins.

6.2. The Possible Glycoprotein Nature of the Putative Verapamil Binding Protein.

Binding of Con-A to proteins immobilised on nitrocellulose paper.

Earlier work using lectin affinity chromatography indicated that the putative verapamil binding protein may not be a glycoprotein. To investigate further the possibility of the verapamil binding protein being glycosylated, an experiment was performed where the purified fractions from each column step in the purification protocol were separated by SDS gel electrophoresis. The separated proteins were then transferred to nitrocellulose paper by western blotting and incubated in peroxidase linked Con-A. The binding of Con-A to the immobilised proteins was detected by assay

for peroxidase activity, with purple bands indicating the presence of glycosylated proteins.

Fig.6.1. shows the binding of Con-A to proteins immobilised on nitrocellulose paper. The lectin is seen to bind to a large number of bands in the DEAE fraction, including a band of Mr 169,000. This suggests that the putative verapamil binding protein may be glycosylated, although the specificity of detection is questionable. Binding of the lectin to proteins from the later stages of the purification (S400 and phenyl Sepharose) was not detected.

6.3. Is the Putative Verapamil Binding Protein a Calcium Binding Protein?

Binding of $^{45}\text{Ca}^{2+}$ to proteins immobilised onto nitrocellulose paper.

To investigate whether the putative verapamil binding protein or other associated proteins in fractions from the final purification steps, were able to bind calcium, nitrocellulose blots of the separated proteins were overlayed with $^{45}\text{Ca}^{2+}$ and the blots autoradiographed. The results suggest that the putative verapamil binding protein does not bind calcium and that there are no detectable associated calcium binding proteins present in the purified fractions.

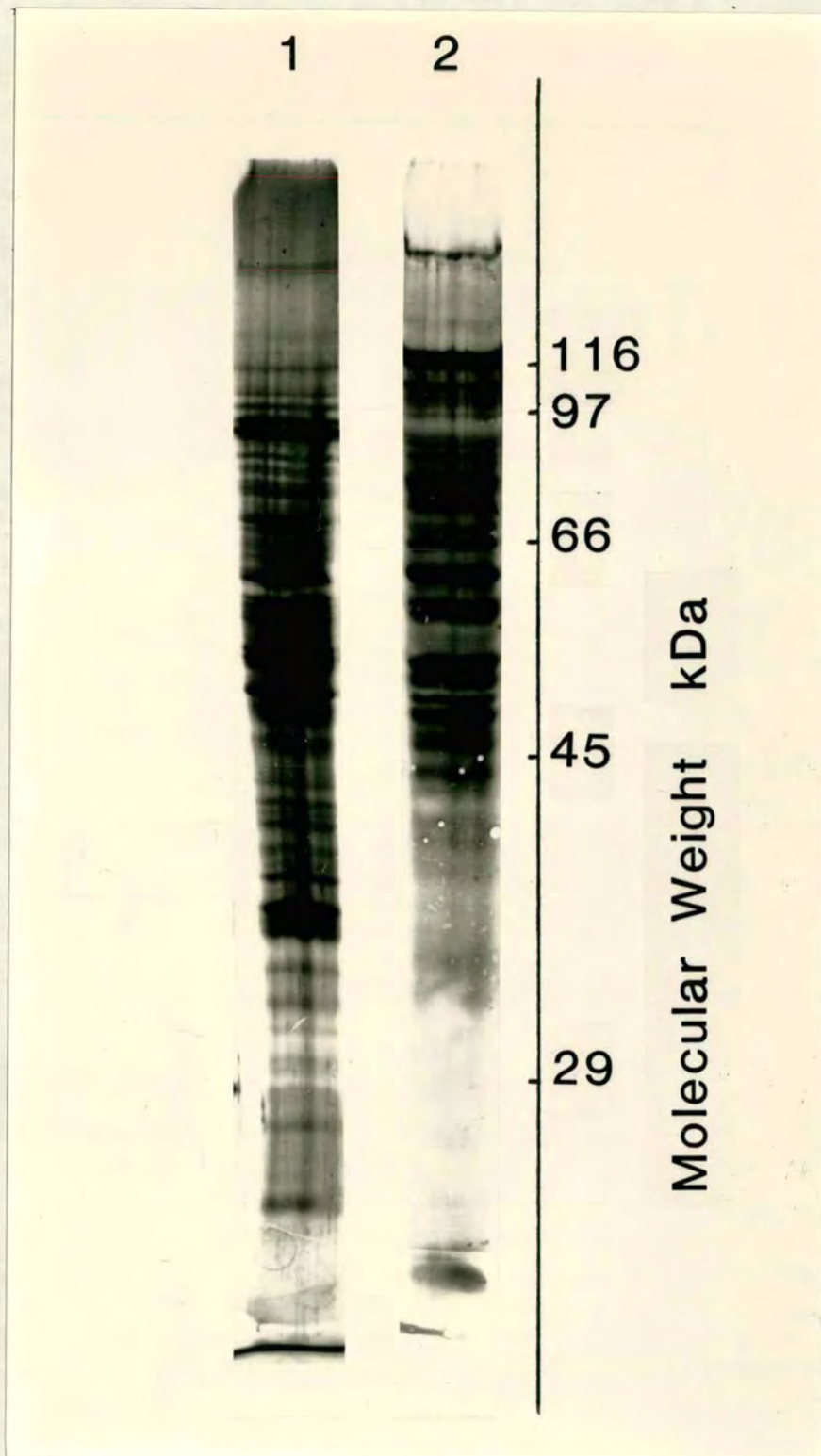


Fig.6.1. Binding of Con-A to peak verapamil binding DEAE fractions separated by SDS PAGE and blotted onto nitrocellulose paper.

Lane 1. Silver stained SDS PAGE separation of a DEAE fraction containing peak verapamil binding activity.

Lane 2. Binding of Con-A to the separated proteins immobilised on nitrocellulose. The dark bands indicate the presence of glycosylated proteins.

6.4. The possible control of plant calcium channels by phosphorylation.

Is the putative verapamil binding protein phosphorylated by endogenous protein kinase(s)?

To investigate the possible role of phosphorylation in the regulation of plant calcium channels, phosphorylation of the putative verapamil binding protein was studied. These investigations focus on the phosphorylation activity of an endogenous kinase(s) which appears to co-purify with the verapamil binding protein. The activity of exogenous kinases such as cAMP-dependent and calcium calmodulin-dependent protein kinases was not studied.

In initial experiments, endogenous kinase activity was detected in crude membrane, DEAE and S400 purified verapamil binding fractions. Table.6.1. shows kinase activity in these different fractions as ATP incorporated in fmol/ μ g protein after 1 minute. The increase in activity over the blank suggests that phosphorylation has taken place. After each purification step the specific activity decreases, which suggests that a kinase may not co-purify with the putative verapamil binding protein or that the substrates for the kinase are being lost as purification proceeds.

Fig.6.2. indicates protein kinase activity over time in DEAE fractions containing peak verapamil binding activity in the absence and presence of calcium. The

<u>Condition.</u>	<u>Phosphate incorporated (fmol/μg of protein).</u>		
	<u>Crude membrane.</u>	<u>DEAE fraction.</u>	<u>S400 fraction.</u>
<u>-Ca²⁺</u>	6.7	6.3	2.1
<u>+Ca²⁺</u>	155.0	5.9	2.3
<u>+Ca²⁺ & CAM</u>	29.5	5.9	2.6
<u>Blank (no protein).</u>	0.3	1.4	1.4

Table 6.1. Endogenous protein kinase activity in crude membrane and column fractions containing peak verapamil binding activity.

Crude membrane or column fractions were incubated in an equal volume of buffer A (+Ca²⁺), buffer B (-Ca²⁺) or buffer C (Ca²⁺ plus Calmodulin). 8 nM ATP was added and after 1 min the phosphate incorporated was measured using the cellulose disc assay (crude membrane) or the phosphocellulose strip assay (column fractions).

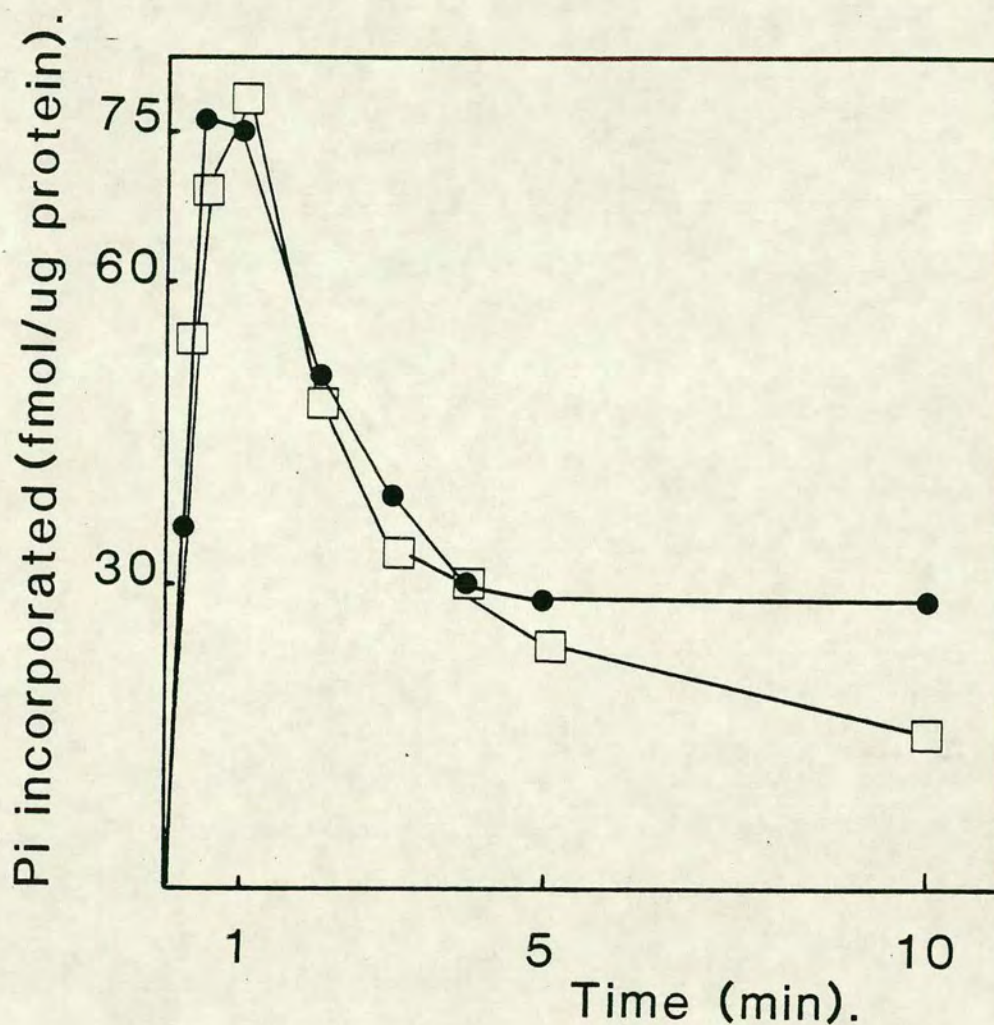


Fig.6.2. Endogenous protein kinase activity in DEAE fractions containing peak verapamil binding activity.

15 μ l of DEAE fraction was mixed with an equal volume of buffer A (+Ca²⁺), (●) or buffer B (-Ca²⁺), (□). 8 nM [γ -³²P] ATP was added and at the time points indicated phosphate incorporation was measured using the phosphocellulose strip assay.

similarity between the graphs suggests that there is little or no calcium dependence of phosphorylation. At the minute time point, kinase activity in the presence of calcium and calmodulin was not significantly different to that seen in the presence of calcium alone, suggesting that kinase activity was also not calcium/calmodulin-dependent (Table.6.1.). The graphs show a peak in phosphate incorporation at 1 minute. Thereafter there is a rapid decline in incorporation to a plateau after about 5 min. The turnover of label suggests that protein phosphatase activity may also be present. The decline of phosphate incorporation may therefore represent the balance between kinase and phosphatase or the action of the phosphatase alone after depletion of the substrate. To investigate this, phosphorylation assays were conducted in the presence of μM ATP concentrations instead of nM ATP concentrations (as used initially), to prevent substrate depletion, plus or minus sodium fluoride, a phosphatase inhibitor.

In the presence of a higher concentration of ATP, phosphate incorporation remains high after 1 min (Fig.6.3.). This suggests that kinase activity is not limited by the substrate. The final plateau level probably represents a balance between kinase and phosphatase activity.

The inclusion of sodium fluoride in the assay mixture would be expected to cause an increase in activity above that seen in the absence of sodium

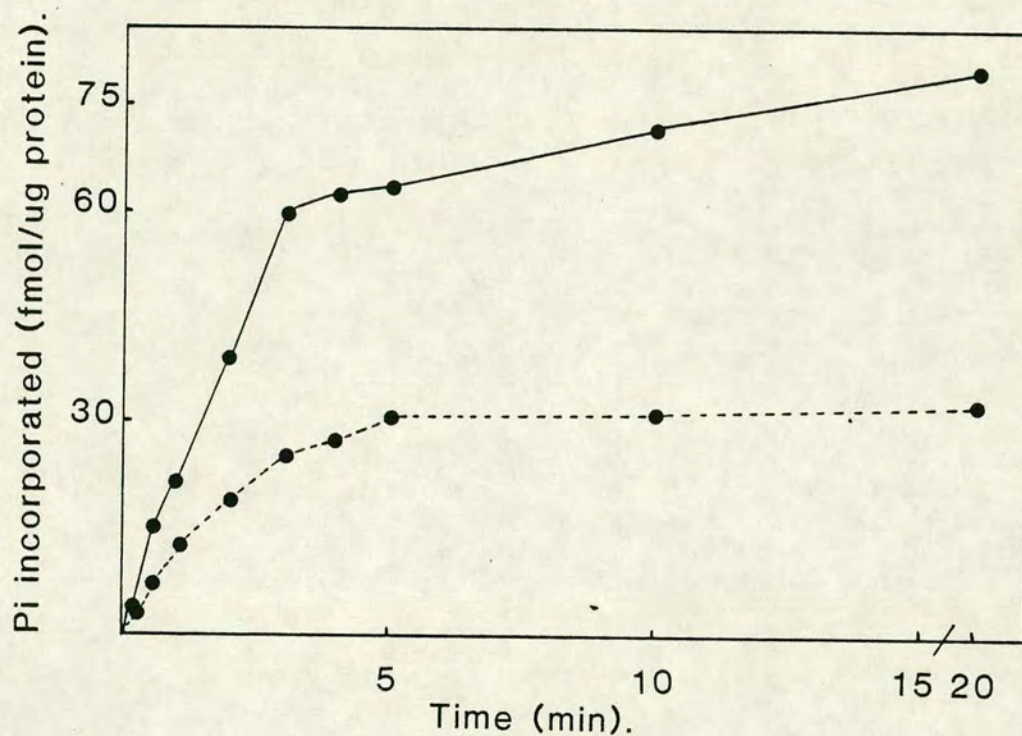


Fig.6.3. Endogenous protein kinase activity in DEAE fractions containing peak verapamil binding activity, measured in the presence of 10 mM sodium fluoride and 1 μ M ATP.

15 μ l of DEAE fraction was mixed with an equal volume of buffer A (+Ca²⁺), plus 1 μ M ATP (●—●) or 1 μ M ATP and 10 nM NaF (●---●). At the time points indicated incorporation of phosphate was measured using the phosphocellulose strip assay.

fluoride. However this is not seen in the graph, which suggests instead that the sodium fluoride is having a non-specific inhibitory effect. The effect of sodium fluoride on kinase activity was not examined.

Autoradiography of phosphorylated partially purified verapamil binding fractions suggests that the putative verapamil binding protein is not phosphorylated by the endogenous protein kinase. Other proteins in the solubilised and DEAE fractions appear to be phosphorylated (Fig.6.4.). Phenyl Sepharose and S400 fractions were not analysed.

6.5. Discussion.

Results from the Con-A blot experiment suggest that the verapamil binding protein may be glycosylated. Con-A binding was only detected to proteins from the DEAE fraction and not to proteins from later stages of the purification. This was probably due to insufficient levels of purified protein (total protein) being blotted onto the nitrocellulose paper. Alternatively, the specific sugar residues required for binding of Con-A may have been lost during purification. This might also explain why lectin affinity chromatography was not successful in purifying the verapamil binding protein. At present it is difficult to draw any firm conclusions about the glycoprotein nature of the putative verapamil binding protein from plant membranes, especially as the Con-A binding assay tends to be rather non-specific.

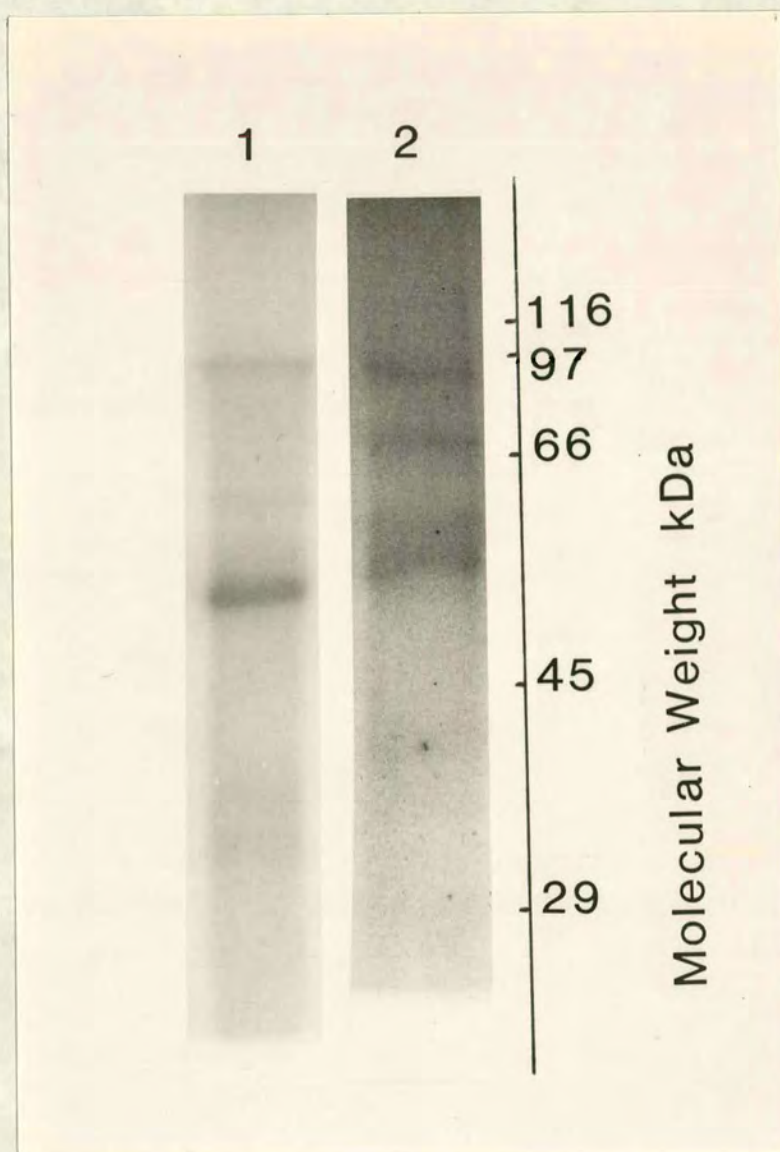


Fig.6.4. Autoradiograph of a phosphorylated solubilised membrane fraction and peak verapamil binding DEAE fraction.

Solubilised membrane fractions and DEAE fractions containing peak verapamil binding activity were incubated in buffer A (+Ca²⁺) and 8 nM ATP for 1 min. Samples were then prepared and separated by SDS PAGE. The gel was then stained with Coomassie blue, dried and autoradiographed.

Calcium binding by the verapamil binding protein and/or any associated channel proteins was not detected. If the plant calcium channel is similar to the skeletal muscle T-tubule channel, then perhaps one might expect calcium binding proteins to be found. However, although the channel needs to be specific for calcium ions, (a specificity apparently achieved by the channel binding calcium), the binding and release of calcium is probably so rapid that it cannot be detected by the $^{45}\text{Ca}^{2+}$ overlay technique. Further studies are required to verify the calcium binding capacity of the putative verapamil binding protein and associated proteins before any conclusions can be drawn about this character of plant calcium channels.

Several conclusions can be drawn from the preliminary phosphorylation studies. 1. There is endogenous kinase activity in the partially purified verapamil binding fractions, but probably not in the final purified fraction, although low protein levels make this difficult to judge properly. 2. The endogenous kinases(s) does not appear to phosphorylate the putative verapamil binding protein, although it does appear to phosphorylate other proteins present in the partially purified fractions. It is possible that phosphorylation of the verapamil binding protein is not detected because it has been phosphorylated already. Further work to clarify the situation in respect to phosphorylation of

the verapamil binding protein needs to be carried out using exogenous kinases.

CHAPTER 7.

DISCUSSION.

7.1. The Experimental Approach: Problems and Criticisms.

7.1.1. *Using [³H] verapamil as a marker for calcium channels.*

Calcium channel antagonists such as nifedipine and verapamil are extremely useful in the study of calcium channels as they bind to specific receptors located within the calcium channel itself. Radiolabelled forms of the drugs can therefore be used to specifically label calcium channel proteins and provide a relatively easy method for calcium channel detection and identification. The labelled channel antagonists have also been used in the purification of the drug binding subunit by either purification of an antagonist-channel complex (Curtis & Catterall, 1984) or by direct binding assay after each purification step (Borsotto et al., 1985). Using these methods the receptors for both the dihydropyridine and phenylalkylamine calcium antagonists plus their associated subunits, i.e. the whole calcium channel have been purified from skeletal muscle.

In this research the calcium channel antagonist [³H] verapamil was used to detect and subsequently purify a verapamil binding protein (presumably the calcium channel), from maize coleoptile tissue. Using a filter assay, [³H] verapamil binding was detected in crude membrane fractions prepared from maize coleoptiles, maize

roots and zucchini hypocotyls. Binding constants for [^3H] verapamil were of slightly lower affinity than those obtained in animal tissue. The results of the [^3H] verapamil binding experiments suggested that calcium channels were present in plant tissues, assuming the antagonist was behaving in the same way as it would in animal tissue. In this thesis no experiments were performed to show whether verapamil causes the blockade of the calcium movement into a cell. However, recently the phenylalkylamine D888 has been shown to inhibit $^{45}\text{Ca}^{2+}$ influx into carrot protoplasts (Graziana *et al.*, 1987). It is interesting to note that although verapamil and other phenylalkylamines have nanomolar binding constants *in vitro*, micromolar to millimolar concentrations are required to produce inhibitory effects on calcium metabolism in tissue or whole plant experiments. This indicates a discrepancy between *in vitro* and *in vivo* concentrations required to obtain verapamil effects. However higher concentrations of verapamil are probably necessary to cause inhibition in intact tissues, as much of the drug may be sequestered or access to the binding sites may be restricted. In this respect, verapamil is thought to bind to the inner mouth of the calcium channel, having gained access to the sites while the channel was open (Hescheler *et al.*, 1982).

7.1.2. Purification of the verapamil binding protein.

Partial purification of the verapamil binding protein was achieved using detergent solubilisation of the membrane bound protein(s), followed by ion exchange, gel filtration and hydrophobic interaction chromatography. SDS polyacrylamide gel electrophoresis of the purified fractions indicated the presence of two major polypeptides of molecular weights 169 kDa and 70 kDa. The 169 kDa polypeptide was favoured to be the verapamil binding protein for two main reasons. 1. The 169 kDa band was found to be absent in non-verapamil binding purified fractions. 2. The 169 kDa band is very similar in size to the α_1 subunit of the L-type calcium channel (155-200 kDa), which is known to contain the phenylalkylamine and dihydropyridine receptor sites (Striessnig *et al.*, 1986b, 1987).

The verapamil binding subunit could not be further accurately identified despite the use of either photoaffinity labelling or [^3H] verapamil overlay techniques.

7.2. The need for calcium channels in plants.

In animal cells a large calcium concentration gradient exists across the plasma membrane, but the permeability of the plasma membrane to calcium is relatively low. Therefore if calcium channels situated on the plasma membrane (and possibly the endoplasmic

reticulum) open, large quantities of calcium ions move rapidly into the cell, down the steep calcium concentration gradient. As a result there is a transient rise in cytoplasmic calcium concentration which can be used as a signal to trigger a cellular response. Calcium channels therefore provide the means for rapid but controlled entry of calcium ions into the cytoplasm, so allowing the modulation of calcium dependent processes.

A similar situation appears to exist in plant cells, which maintain a low cytosolic calcium concentration against a high concentration of calcium in the apoplast. Controlled entry of calcium via a channel has the same potential to act as a signal or a second messenger.

Calcium channels have a central role in this type of signalling system, but there is only limited evidence for their existence in plants. In a number of studies, calcium channel antagonists have been shown to bind to various plant membranes. For example, verapamil binding has been demonstrated in zucchini hypocotyl and maize coleoptile membranes by the author and others (Andrejauskas *et al.*, 1985). Binding of nitrendipine to pea seedling membranes has been shown by Hetherington & Trewavas (1984) and recently D888 has been demonstrated to block $^{45}\text{Ca}^{2+}$ influx into carrot protoplasts (Graziana *et al.*, 1987). Calcium channel antagonists have also been used to inhibit various developmental events in plants. For example, cytokinin-induced bud formation is inhibited by D600 and verapamil (Saunders & Hepler, 1983);

metaphase is inhibited by D600 (Hepler, 1985), polarised growth in *Micrasterias* is inhibited by verapamil (Lehtonen, 1984) and tip growth of pollen tubes is inhibited by nifedipine (Reiss & Herth, 1985).

As well as these inhibitor studies, the similarity in size of the putative verapamil binding protein (169 kDa) found in this research, compared to the main functional subunit of the voltage-dependent calcium channel from skeletal muscle (155-200 kDa), (Striessnig *et al.*, 1986a), is yet another piece of evidence to suggest that calcium channels exist in plant cells.

7.3. What are the functions of plant calcium channels?

Calcium channel antagonist studies suggest that calcium channels are important in the co-ordinated growth and development of plants. Once calcium moves inside the cell via the calcium channel, the calcium concentration can vary spatially and temporally. These variations result in calcium concentration gradients and localised areas of calcium which appear to influence the development pattern of the cell. Brownlee & Wood, (1986), demonstrated the presence of a longitudinal gradient of cytosolic calcium in the rhizoid cells of *Fucus serratus*. Their results suggested that Ca^{2+} enters the rhizoid tip through calcium channels. They postulated that this gradient was important to cell polarity and growth of the cell. Ca^{2+} gradients have also been found in other tip growing cells, for example Lily pollen tubes (Reiss &

Herth 1985). Saunders (1986) has shown that calcium channels are involved in the establishment of a gradient in *Funaria* which determines the subsequent growth pattern. In electrophysiological studies and the use of Fura-2 AM, cytokinin has been demonstrated to induce an increase of inward calcium current along the length of *Funaria* cells, which results in a high free calcium zone at the distal ends of the cell i.e. the presumptive bud site. From these results it was postulated that cytokinin activates plasma membrane located calcium channels that subsequently create a zone of high calcium at the site of the new bud. This study indicates that calcium channels may be involved in the localised development of plant cells. Localised influx of calcium via the calcium channel may also provide the cell with spatial and positional information for cytoskeletal assembly and structure (Brawley & Robinson, 1985).

The processes described above tend to require the presence of calcium for several hours before a response is induced. Calcium channels therefore, may be involved in maintaining elevated calcium levels in plant cells. As well as these 'slow' responses, the presence of calcium channels helps to explain more transient changes or 'fast' responses. Such responses include chloroplast movement in *Mougeotia* which is a light-dependent, calcium-mediated response (Wagner *et al.*, 1984), red-light dependent germination of *Onoclea* spores (Wayne & Hepler, 1984) and the action potential dependent increase

of cytoplasmic calcium concentration in *Chara* which inhibits cytoplasmic streaming (Williamson & Ashley, 1982).

7.4. Properties of Plant Calcium Channels.

As there is limited data on calcium channels in plants, it is difficult to make generalisations about their properties. In particular, whether there are families of calcium channels, whether they are localised according to membrane type and how they are controlled, i.e. are they voltage-dependent and/or receptor operated?

In animal cells families of calcium channels have been distinguished on the basis of their differing sensitivities to various calcium channel antagonists (Glossmann *et al.*, 1982). It is possible that the differential binding characteristics of verapamil, D888, D600 and nifedipine seen in this research, may reflect different families of channels, each sensitive to a different antagonist. Nifedipine for example, was unable to displace verapamil in this research and did not inhibit $^{45}\text{Ca}^{2+}$ influx into carrot protoplasts (Graziana *et al.*, 1987). However nifedipine has been shown to affect pollen tube growth (Reiss & Herth, 1985) and *Fucus* zygote polarity (Brownlee & Pulsford, 1988).

The cellular location of calcium channels in plants has not been rigorously examined. Preliminary data presented in this thesis indicates increased verapamil binding (and therefore presumably calcium channels) in

plasma membrane enriched fractions compared to fractions containing the other membrane types. This parallels work by Andrejauskas *et al.* (1985), which also indicated high verapamil binding in plasma membrane fractions, with slightly less in fractions of endoplasmic reticulum and tonoplast. These results suggest that plant calcium channels are localised according to membrane type. It is possible that channel sensitivity to channel antagonists may vary according to their location, which might be a useful tool to identify and purify the various channel types from different membranes.

In animal cells calcium channels open either when the membrane is depolarised (voltage-dependent channels) or when a ligand binds to the channel (receptor-operated channels). Whilst the requirements for the opening and closing of plant calcium channels are not definitely known, there is evidence for voltage-dependent ion channels in plant cells, in particular the tonoplast (Hedrich & Neher, 1987; Hedrich & Schroeder, 1988), indicating that change in membrane potential can be used to control ion entry. Other pieces of evidence also point to the existence of voltage-dependent calcium channels in plants; a calcium action potential has been demonstrated in *Chara*, which is thought to trigger the opening of calcium channels thereby increasing the cytoplasmic calcium concentration (Williamson & Ashley, 1982); calcium flux into corn root tissue was found to increase as a result of actions which depolarised the membrane

(Rincon & Hanson, 1986). At present there is little firm evidence for receptor-operated channels in plant cells.

7.5. Future Work.

Information about calcium channels in plants is limited. As this discussion indicates, there are many questions that still remain to be answered. In general terms these questions cover all aspects of calcium channel behaviour and control. More specifically the research presented in this thesis needs to be taken further and some of the findings investigated in more depth. This would include making a definite identification of the 169 kDa protein which is proposed to be the verapamil binding protein. This may involve further photoaffinity labelling experiments, although so far these have been unsuccessful.

Further verification that we are dealing with a calcium channel protein may come from antibody studies, although it has been shown that monoclonal and polyclonal antibodies developed for dihydropyridine and phenylalkylamine receptors from skeletal muscle do not cross react with plant membranes. However antibodies, raised to the 169 kDa protein, could be used to demonstrate the presence of channel proteins by the inhibition of $^{45}\text{Ca}^{2+}$ influx into protoplasts. The antibodies could also be used to demonstrate the location and clustering of channels in histochemical studies,

which could then be related to calcium influx by the use of dyes and ratio imaging technology.

A molecular approach could also be taken to elucidate the identity of the 169 kDa protein. The protein could be sequenced and compared to that of a known calcium channel; the sequence for the α_1 subunit of the voltage-dependent calcium channel from skeletal muscle has already been determined (Tanabe *et al.*, 1987).

Ultimately to determine if the putative verapamil binding protein is a functional channel, the protein could be reconstituted into lipid bilayers and then patch clamped. Depolarisation of the membrane and the addition of calcium channel antagonists would determine if the protein is a functional channel. One drawback to this approach, is that for the isolated verapamil binding protein to act as a functional channel may require the presence of its' associated subunits (assuming that the plant calcium channel does consist of a number of subunits).

The control and regulation of plant calcium channels is an extremely interesting area for further research. Animal calcium channels are known to be regulated by cAMP-dependent and Ca^{2+} calmodulin-dependent protein kinases and possibly protein kinase C (O'Callahan, 1988). Phosphorylation may also be important in the regulation of plant calcium channels and to investigate this an electrophysiological and/or biochemical approach could be taken. As in the animal channel work, the activity of

exogenous protein kinases on the purified antagonist binding protein could be studied, as a continuation of the studies made on the endogenous kinase activity presented in this thesis.

Possibly one of the main ways to discover more about plant calcium channels will be by patch clamping. This technique has been successfully applied to plant protoplasts and has helped to elucidate more about the mechanisms of ion transport (Hedrich *et al.*, 1987). Using patch clamping the properties of a single channel, such as ionic selectivity, conductance, kinetics and modulation by chemicals and drugs can be studied. Using a combination of patch clamping and for example intracellular calcium dyes, it might be possible to determine whether calcium does enter the cell via the calcium channel or is released from intracellular stores in response to external stimuli. So far there have been no reports of plasma membrane calcium channels found by patch clamping, (although there may only be very few per cell which might make them very difficult to find). However patch clamping has identified two types of calcium-dependent ion channels in the vacuolar membrane, which might conceivably be calcium channels. Patch clamping is a powerful technique for ion channel study and may provide, in combination with biochemical and molecular biological techniques, the way forward in plant calcium channel research.

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APPENDIX.

Partial purification of a protein from maize (*Zea mays*) coleoptile membranes binding the Ca^{2+} -channel antagonist verapamil

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A protein that binds the calcium-channel antagonist verapamil has been partially purified from maize (*Zea mays*) coleoptile membranes. The protein was solubilized with the detergent CHAPS [3-(3-cholamidopropyl)dimethylammonio]propane-1-sulphonate] and purified by a combination of ion-exchange, gel-filtration and hydrophobic-interaction chromatography. This resulted in a 120-fold purification. SDS/polyacrylamide-gel-electrophoretic analysis of the polypeptides from the final purification step indicated that the verapamil-binding protein may have a major component of M_r 169 000. The dissociation constants for specific binding of [^3H]verapamil to crude and CHAPS-solubilized maize coleoptile membrane fractions are 72 nM and 158 nM respectively, with respective binding-site concentrations of 135 pmol/mg of protein and 78 pmol/mg of protein. In both cases the Scatchard plots are linear, indicating a single class of binding sites. [^3H]Verapamil binding to crude maize coleoptile membrane fractions could not be displaced by unlabelled desmethoxyverapamil or by nifedipine, but could be displaced by unlabelled methoxyverapamil.

INTRODUCTION

Ca^{2+} is a ubiquitous regulator of growth and development [1]. It is known, that there are similarities between the control systems involving Ca^{2+} in both plants and animals [2-6]. Plant cells regulate their cytosolic Ca^{2+} and maintain it at a submicromolar concentration. Active-transport systems pump Ca^{2+} out of the cell or into organelles. Calmodulin has been isolated from numerous plants and tissues. Changes in cytosolic Ca^{2+} are believed to contribute to the control of diverse phenomena such as tropic bending, stomatal closure and germination.

The entry of Ca^{2+} into plant cells have received little attention. In animal cells (neurons, skeletal muscle and cardiac muscle), voltage-dependent Ca^{2+} channels are an important route for Ca^{2+} entry into excitable cells. They are necessary for smooth-, cardiac- and skeletal-muscle contraction and for secretory processes [7-9]. Ca^{2+} -channel antagonists are a group of drugs (used to treat cardiovascular disorders) of which there are three main structurally unrelated types. These are the dihydropyridines, e.g. nifedipine, the phenylalkylamines, e.g. verapamil, and the benzothiazepines, e.g. diltiazem [10,11]. These compounds bind to specific receptors located in the Ca^{2+} channel. The radioactive forms of the channel antagonists can be used to label specifically the Ca^{2+} -channel proteins, so providing a means of Ca^{2+} -channel detection and identification [12,13].

Experimental evidence suggests that Ca^{2+} channels do exist in plant cells [6,14,15]. Ca^{2+} -channel antagonists have been demonstrated to disrupt a number of plant functions [16-18]. Nifedipine and verapamil have been shown to bind specifically to plant membranes *in vitro* [19-21]. More recently, various phenylalkylamines have been shown to inhibit $^{45}\text{Ca}^{2+}$ influx into carrot (*Daucus*

carota) protoplasts [2]. A direct relationship has been established between membrane binding constants for range of phenylalkylamines and the ability of the drug to inhibit Ca^{2+} uptake [22].

Here we present data for [^3H]verapamil binding to maize (*Zea mays*) coleoptile membranes and describe a method for the solubilization and partial purification of the verapamil-binding protein from maize coleoptile membrane preparations.

MATERIALS AND METHODS

Plant material

Maize (*Zea mays* cv. Leader) seeds, from the National Institute of Agricultural Botany, Cambridge, U.K., were grown in moist vermiculite in the dark at 25 °C. After 5 days the coleoptiles, including the leaf rolls, were harvested and collected on ice.

Chemicals

[*N*-methyl- ^3H]Verapamil hydrochloride, with a specific radioactivity of 68.8 Ci/mmol, was obtained from New England Nuclear, Bedford, MA U.S.A. Unlabelled (+/-)-verapamil hydrochloride was obtained from Sigma. All other chemicals were of the highest grade available, obtained from various sources.

Preparation of crude membrane fractions

All preparative procedures were carried out at 4 °C unless stated otherwise. Freshly harvested tissue was chopped with a razor blade and then homogenized in (2 vol. per g fresh weight) 50 mM-Tris/3 mM-EDTA buffer, adjusted to pH 7.5 with HCl. The homogenate was then filtered through one layer of nylon cloth and centrifuged at 4000 g for 20 min. The pellet was discarded

Abbreviations used: PEG, poly(ethylene glycol); PLE, poly(ethyleneimine); CHAPS, [3-(3-cholamidopropyl)dimethylammonio]propane-1-sulphonate; PAGE, polyacrylamide-gel electrophoresis; DTT, dithiothreitol; Con A, concanavalin A; IC_{50} , inhibition constant (concentration at which 50% of the specifically bound radioligand is displaced); B_{max} , maximum concentration of binding sites.

and the supernatant re-centrifuged at 50000 g for 45 min, yielding the crude membrane pellet, which was resuspended in 50 mM-Tris/50 mM-Mops buffer, adjusted to pH 7.5 with HCl, by using a glass/Teflon homogenizer.

Solubilization of the crude membrane fraction

Crude membrane preparations were incubated at a protein/detergent ratio of 1:14 (w/w), in 0.5% (w/v) CHAPS/20% (w/v) sucrose/50 mM-Tris/50 mM-Mops buffer, adjusted to pH 7.5 with HCl, for 30 min on ice and then centrifuged for 1 h at 100000 g. The resulting supernatant contained the solubilized proteins.

Verapamil-binding assay

In the standard assay, [3 H]verapamil was added to the crude membrane resuspension, solubilized membrane fraction or column fraction, to give a final concentration of 2.24 nM. Unlabelled verapamil or other compounds were then added as required to a final assay volume of 500 μ l. After an incubation period of 120 min at 20 °C, the reaction was stopped by the addition of 3 ml of ice-cold wash buffer containing 10% (w/v) PEG 8000/50 mM Tris, adjusted to pH 7.5 with HCl. The mixture was immediately filtered under vacuum through Whatman GF/C filters (pretreated with 0.3% PFI solution for at least 2 h, when solubilized fractions were assayed), using a Millipore multifilter unit. The filters were washed once with 3 ml of ice-cold wash buffer. The radioactivity retained on the filters was determined by liquid scintillation counting. Non specific binding was determined by incubating samples with 100 μ M unlabelled verapamil and was subtracted from total binding (obtained from assays containing [3 H]verapamil only), to give the specific binding of [3 H]verapamil.

Partial purification of the verapamil-binding protein

DEAE-Bio-Gel ion-exchange chromatography. Solubilized membrane fraction from approx. 100 g fresh weight of tissue was loaded on to a column (4.0 cm \times 1.0 cm) of DEAE-Bio-Gel A (Bio-Rad), which had been equilibrated with column buffer containing 50 mM-Tris, 50 mM-Mops, 20% sucrose, 0.1% CHAPS, pH 7.5 (adjusted with HCl). The ratio of sample to matrix was 5:1 (v/v). After loading of the sample, the column was washed with an equal volume of column buffer. Elution with 0.15 M-NaCl in column buffer yielded the verapamil-binding activity in the first 8 ml of the eluate. This was collected, portions were assayed directly for verapamil binding, and the eluate then quick frozen in liquid N₂ and stored at -80 °C until required for further purification.

Sephaeryl S-400 gel filtration. A Sephaeryl S-400 column (with a bed volume of 38.8 ml) was equilibrated with column buffer at a flow rate of 0.5 ml/min. The post-DEAE-Bio-Gel fraction (2 ml) was loaded on to the column and eluted with column buffer. Fractions (1 ml each) were collected, and portions were assayed for verapamil-binding activity. Fractions with the highest binding activity were pooled.

Phenyl-Sepharose hydrophobic-interaction chromatography. Pooled fractions from the Sephaeryl column were made 1 M with respect to (NH₄)₂SO₄ and 1 mM with respect to CaCl₂. They were loaded on to a column (2.0 cm \times 1.0 cm) of phenyl-Sepharose CL-4B

(Pharmacia) that had been equilibrated with 10 column volumes of buffer containing 50 mM-Tris, 50 mM-Mops, 20% sucrose, 0.1% CHAPS, 1 M-(NH₄)₂SO₄, 0.1 mM-CaCl₂, pH 7.5 (adjusted with HCl) (buffer A). After washing with buffer A, the column was eluted with 18 ml each of the following series of buffers: B, 50 mM-Tris/50 mM-Mops/20% sucrose/0.1% CHAPS/0.1 mM-CaCl₂, pH 7.5 (adjusted with HCl); C, 50 mM-Tris/50 mM-Mops/20% sucrose/0.1% CHAPS, pH 7.5 (adjusted with HCl); D, 0.1% CHAPS/20% sucrose, pH unadjusted. Fractions (2 ml) were collected and 1 ml samples were assayed for verapamil binding.

SDS/PAGE

Samples were denatured in 2.35% (w/v) SDS/10% (v/v) glycerol/62.5 mM-Tris/HCl (pH 6.8)/5% (v/v) β -mercaptoethanol for 10 min at 95 °C and separated by PAGE on 10% (w/v) polyacrylamide gels, using the buffer system of Laemmli [23]. Gels were stained with silver [24]. Molecular-mass markers obtained from Sigma were: myosin (200 kDa), β -galactosidase (116 kDa), phosphorylase b (92 kDa), bovine serum albumin (66 kDa) and carbonic anhydrase (31 kDa).

Protein determination

This was carried out by a modified Bradford [25] method, with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Kinetics of [3 H]verapamil binding to maize coleoptile membranes

Maize coleoptile membranes (crude and CHAPS-solubilized) bind verapamil in a saturable manner, and this binding is reversible. Binding equilibrium is reached after approx. 120 min (Fig. 1). When 100 μ M unlabelled verapamil is added at the 'plateau', dissociation of bound verapamil is initiated and is largely complete after a further 120 min. Similar kinetic data have been found

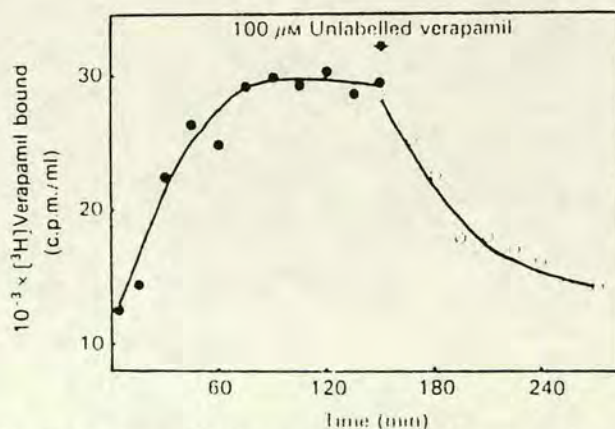


Fig. 1. Kinetics of [3 H]verapamil binding to maize coleoptile membrane fractions

Association was initiated by addition of [3 H]verapamil (final concn 1.12 nM) to the incubation mixture containing membrane protein (0.38 mg/500 μ l assay volume). At the times indicated, 250 μ l samples were withdrawn and assayed as described in the Materials and methods section. Dissociation was initiated by addition of 100 μ M-verapamil.

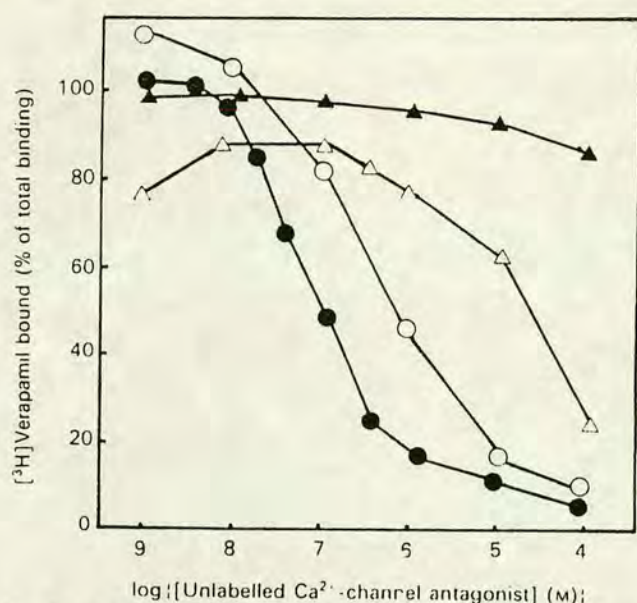


Fig. 2. Displacement of [^3H]verapamil by unlabelled Ca^{2+} -channel antagonists

Binding of [^3H]verapamil to maize coleoptile membrane fractions was measured under equilibrium conditions, at pH 7.5 and 20°C , in the presence of either unlabelled verapamil (\bullet), methoxyverapamil (\circ), desmethoxyverapamil (\triangle) or nifedipine (\blacktriangle) at the concentrations indicated. Total verapamil binding without addition of unlabelled compounds was 250 fmol, and non-specific binding (determined by the addition of $100\ \mu\text{M}$ unlabelled verapamil) was 4% of the total.

for both zucchini (courgette, *Cucurbita pepo*) membranes and skeletal-muscle microsomes [20,26].

Of the drugs tested for displacement of [^3H]verapamil, only verapamil and methoxyverapamil were able to appreciably displace [^3H]verapamil binding in maize coleoptile membranes, with IC_{50} values of 100 nM and 700 nM respectively (Fig. 2). Desmethoxyverapamil competed poorly, even though it is closely related to verapamil and has an IC_{50} value of about $50\ \mu\text{M}$. Nifedipine, which is of a different structural class, had little effect, even at $100\ \mu\text{M}$. These results suggest that there are specific binding sites for the different Ca^{2+} -channel antagonists within a channel.

Figs. 3(a) and 3(b) show Scatchard plots for [^3H]verapamil binding in crude and CHAPS-solubilized membrane fractions. For crude membrane fractions they indicated a K_D of 72 nM and of B_{max} (binding-site concentration) of 135 pmol/mg of protein. In the CHAPS-solubilized preparation the K_D was 158 nM and the B_{max} 78 pmol/mg of protein. In both cases the plots are linear, suggesting the presence of a single class of binding sites. The similarities in dissociation constants suggest that the solubilized verapamil-binding protein has properties similar to those of the membrane-bound protein.

Scatchard plots were also constructed for [^3H]verapamil binding to crude membrane preparations from maize roots and zucchini hypocotyls, yielding linear plots in both cases (results not shown). Maize roots were found to have a K_D of 233 nM for verapamil binding and a B_{max} of 165 pmol/mg of protein. A K_D

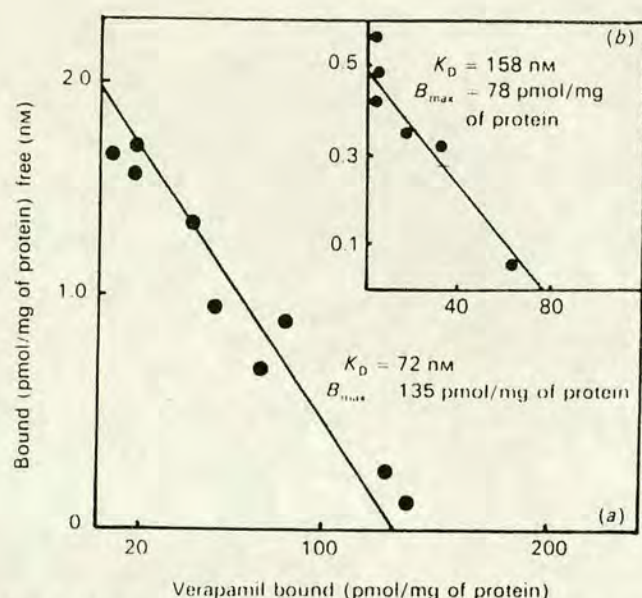


Fig. 3. Scatchard plots of [^3H]verapamil binding in (a) crude and (b) solubilized maize coleoptile membrane fractions

Least-squares regression analysis was used to plot the line of best fit.

of 114 nM and a B_{max} of 96 pmol/mg of protein was obtained for zucchini membrane fractions, which compares favourably with values obtained previously for zucchini, where the K_D was 102 nM and the B_{max} 60 pmol/mg of protein [20]. It is noticeable that the dissociation constants obtained for plant crude membrane fractions are of a lower affinity than those obtained for skeletal-muscle microsomes preparations, for which the K_D was 43 nM and the B_{max} 44 pmol/mg of protein [26]. The concentration of verapamil-binding sites also tends to be greater for plant tissues.

Partial purification of the verapamil-binding protein

The verapamil-binding protein was partially purified from maize coleoptile membranes by using anion-exchange and gel-filtration chromatography, followed by phenyl-Sepharose hydrophobic-interaction chromatography. Lectin-affinity chromatography was attempted but verapamil-binding proteins in solubilized maize coleoptile membrane preparations and DEAE-Bio-Gel fractions did not adhere to Con A Sepharose or to wheat-germ-lectin columns. The phenyl-Sepharose column was used in order to take advantage of the hydrophobic nature of the Ca^{2+} channel.

After initial studies with a variety of detergents, the verapamil-binding sites were optimally solubilized by 0.5% CHAPS at a protein/detergent ratio of 1:14 (w/w). The inclusion of 20% sucrose during solubilization improved the stability of the solubilized verapamil-binding protein. Under these conditions the recovery of verapamil-binding sites was approx. 50%.

The solubilized receptor was then subjected to DEAE-Bio-Gel anion-exchange chromatography. Verapamil-binding activity was eluted with 0.15 M-NaCl in column buffer. The inclusion of 0.1% CHAPS in the elution buffer was found to be necessary for successful elution of the verapamil-binding protein. Under these conditions,

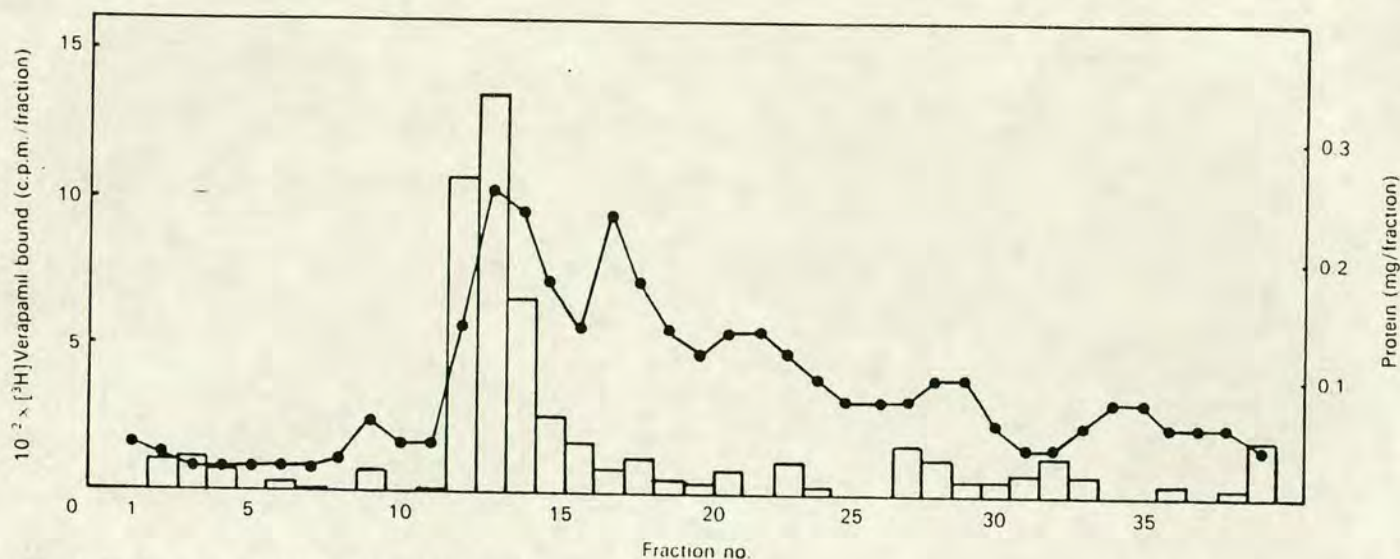


Fig. 4. Elution profile for the Sephacryl S-400 column

Fractions from the DEAE-Bio-Gel ion-exchange column (2 ml) were loaded on to the column (1.5 cm \times 22 cm) of Sephacryl S-400 and eluted as described in the Materials and methods section. The 1 ml fractions were assayed for verapamil binding. The void volume of the column was determined separately with Blue Dextran 2000.

the degree of purification obtained averaged about 3-fold (varying from 1 to 7 fold, for reasons that are not clear). Recovery of binding activity ranged from 20 to 50 %. Although the purification factor achieved with the DEAE-Bio-Gel column is fairly low it appeared to be a necessary step in order that the subsequent purification steps should be successful.

Fractions containing verapamil-binding activity eluted from the DEAE-Bio-Gel column were further fractionated on a Sephacryl S-400 column. A typical elution profile is shown in Fig. 4. The recovery of binding activity was more than 100 %, of that loaded on to the column, perhaps because of removal of proteins inhibitory to binding. The recovery of binding activity was 74 %, of that initially in the solubilized membrane extract. The degree of purification achieved with the gel-filtration step was 76-fold (Table 1). The verapamil-binding protein was eluted in the void volume, suggesting that it chromatographed as a large aggregate with a molecular mass of more than 8000 kDa. The presence of 1 M-NaCl or 1 mM-DTT in the column buffer had no effect on the elution behaviour.

Verapamil-binding fractions from the Sephacryl S-400 column were then applied to a phenyl-Sepharose column. Fig. 5 shows a typical elution profile from the column. The profile indicates that verapamil-binding activity is eluted partly by buffer B, and partly by buffer D, the latter fraction (eluted with 0.1 % CHAPS/20 % sucrose), having the higher specific activity. The recovery of verapamil-binding activity from the phenyl-Sepharose column (buffer D fraction) was 20 %, and overall recovery was less than 4 %, of the initial activity in the CHAPS-solubilized membrane extract.

After each purification step, the proteins contained in the different fractions were analysed by SDS/PAGE (Fig. 6). The final purification step gave four stained bands of M_r 169000, 100000, 70000 and 66000 (Fig. 6, lane 5). Silver staining of the gel indicated a band of M_r 169000 which was enriched in the final verapamil-

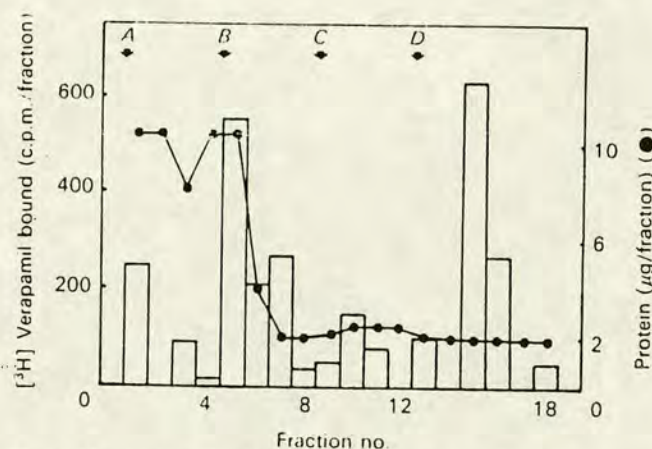


Fig. 5. Elution profile for the phenyl-Sepharose column

Pooled fractions from the Sephacryl S-400 column were adjusted to 1 M-(NH₄)₂SO₄ and 1 mM-CaCl₂ and loaded on to the phenyl-Sepharose column, which had previously been equilibrated with 10 column volumes of Buffer A. Numerous elution protocols were evaluated. The following elution series gave the best recovery of verapamil-binding activity. When sample loading was complete, the column was washed with up to 18 ml of buffer A, and then eluted with 18 ml each of buffers B, C and D (arrows). Fractions (2 ml) were collected, and the first four 2 ml fractions of each elution step were assayed for verapamil binding.

binding fraction (Fig. 6, lane 5, see arrow). This band was absent from the protein profiles of inactive fractions (Fig. 6, lane 4). A protein of M_r 100000 was observed throughout the verapamil-binding and non-verapamil-binding fractions from the Sephacryl S-400 column and the breakthrough fractions from the DEAE-Bio-Gel column. A protein of equivalent M_r 70000, was observed in some of the non-verapamil-binding fractions of the

Table 1. Purification of the verapamil-binding protein

Purification step	Verapamil bound		Protein		Specific activity	Purification (fold)
	(c.p.m.)	(%)	(mg)	(%)		
CHAPS Extract	32240	100	25	100	1305	1
DEAE-Bio-Gel	10304	32	4	16	2525	2
Sephaeryl S-400	23800	74	0.24	0.108	99166	76
Phenyl-Sepharose	1264	4	0.008	0.032	158000	121

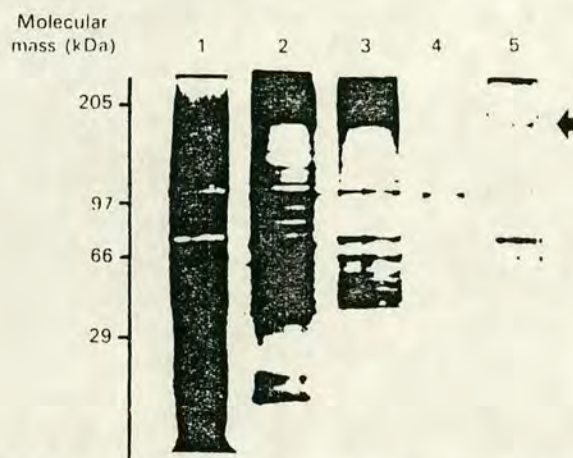


Fig. 6. SDS/PAGE of verapamil-binding fractions from maize coleoptile membranes at different stages of purification

Lane 1, CHAPS-solubilized extract (5 μ g of protein); lane 2, pooled DEAE-Bio-Gel fraction (5 μ g of protein); lane 3, pooled Sephaeryl S-400 fraction (5 μ g of protein); lane 4, Sephaeryl S-400 fraction with no verapamil-binding activity (5 μ g of protein); lane 5, buffer D fraction from the phenyl-Sepharose column (1 μ g of protein).

Sephaeryl S-400 elution profile (Fig. 6, lane 4). The band at 66000 M_r is a staining artefact frequently observed even in unloaded SDS/PAGE slots and reported by others [27]. On this basis the protein of M_r 169000 is the most likely candidate to contain the verapamil-binding site.

The gel suggests that there has been extensive purification of the verapamil-binding protein. However, the purification factor, 120-fold (Table 1), is higher than might be anticipated from a simple calculation of the abundance of the protein in the initial CHAPS extract. Despite care, we have found the binding-site activity to be unstable during purification. This instability, combined with potential inaccuracies in measuring low levels of protein, probably accounts for this.

To investigate the possible glycoprotein nature of the verapamil-binding protein, gels of the post-DEAE-Bio-Gel verapamil-binding fraction were blotted on to nitrocellulose paper and incubated with peroxidase-linked Con A. The lectin was seen to bind to the band of M_r 169000 and to other proteins present in this DEAE-Bio-Gel purified fraction, suggesting that the M_r 169000 component is a glycoprotein. Binding of the lectin to the later stages of the purification was not obtained, possibly owing to insufficient levels of the purified protein.

The M_r 169000 component of the verapamil-binding protein in maize coleoptile membranes found in the present study is comparable with the major subunit of M_r 142000 found for the dihydropyridine receptor in rabbit skeletal muscle and to the polypeptide of M_r 165000 found for the phenylalkylamine receptor (also in rabbit skeletal muscle) [28–30]. If the Ca^{2+} channel has been conserved during evolution, one would expect to see a similarity in the size of the channel polypeptides in plant and animal tissues. Monoclonal and polyclonal antibodies have been developed for dihydropyridine and phenylalkylamine receptors from skeletal muscle [31,32]. However, these antibodies do not cross-react with plant membranes (M. Lazdunski, personal communication) which suggests some structural differences between the receptors in plants and animals.

Further work is needed to determine whether the major band of M_r 169000 obtained from the partially purified maize coleoptile membrane preparations is indeed the phenylalkylamine receptor in plants.

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